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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit: 1648
BLONDER et al.	Examiner: Li, Bao Q.
Serial No.: 09/888,235	
Conf. No.: 8106	APPEAL BRIEF (37 C.F.R. § 41.37)
Filed: June 22, 2001	
Atty. File No.: 42830-00234	
For: "DELIVERY VEHICLE COMPOSITION AND METHODS FOR DELIVERING ANTIGENS AND OTHER DRUGS"	EXPRESS MAIL LABEL NO. EV386652150US

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This Appeal Brief is filed in relation to appeal of the referenced Application from claim rejections stated in a final Office Action of November 16, 2004 (hereinafter, "Final Office Action").

This Appeal Brief includes the following appendices:

Appendix A – Claims;

Appendix B – Evidence; and

Appendix C – Related Proceedings.

Enclosed herewith is a check in the amount of $250 for payment of the fee specified in 37 C.F.R. § 41.20(b)(2) for filing a brief in support of an appeal. No other fees are believed to be due with this communication. If, however, any other fees are due with this communication, please debit such fees to Deposit Account No. 50-1419. To the extent that timely filing of this communication requires an extension of time under 37 C.F.R. § 1.36(a), consider this communication to include a petition for such extension.

REAL PARTY IN INTEREST

The real party in interest is RxKinetix, Inc, a Delaware corporation, assignee of record of the Application.

RELATED APPEALS AND INTERFERENCES

None.

STATUS OF CLAIMS

Claims 1, 4-7, 9-31, 33-37, 39-44, 148, 149 and 150-197 are pending in the application, Claims 2, 3, 8, 32, 38 and 45-147 having been cancelled. Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 are the subject of this appeal. Claims 150-197 are withdrawn from consideration, as further discussed below.

Pending Claims 1, 4-7, 9-12, 15, 43, 44, 148 and 149 are rejected under 35 U.S.C. § 1.02(b).

Pending dependent Claims 13, 14, 16-31, 33-37 and 39-42 are listed as being rejected on the Office Action Summary of the Final Office Action, although no basis for rejection is specifically stated in the Final Office Action with respect to those Claims.

Through an inadvertence during prosecution, Claims 150-197, which are withdrawn from consideration, are technically still pending in the application, even though those claims are not listed in the Final Office Action. During prosecution, these claims were inadvertently dropped from the claim listing. There are no issues to address on appeal with respect to Claims 150-197, and an amendment has been submitted requesting cancellation of those claims, as discussed in the next section below.

STATUS OF AMENDMENTS

An amendment was filed pursuant to 37 C.F.R. §§ 41.33 &1.116 on June 20, 2005 requesting cancellation of Claims 150-197. At this time, it is believed that the amendment has not been entered.

SUMMARY OF CLAIMED SUBJECT MATTER

The claims subject to appeal are directed to a composition for delivery of an antigen (Claims 1, 4-7, 9-31, 33-37, 39-43, 148 and 149) and to a method of packaging and storing such a composition (Claim 44). (*See, inter alia*, specification generally at page 6, line 27, through page 9, line 19 and at page 10, lines 26-28.)

Claim 1, the only independent claim at issue in this appeal, is directed to a composition for delivery of an antigen, and recites the following components and features:

0.0001 weight percent to 5 weight percent of an antigen (*see, inter alia*, specification at page 15, lines 13-22 and page 20, lines 25-29);

5 weight percent to 33 weight percent polyoxyalkylene block copolymer that is biocompatible (*see, inter alia*, specification at page 17, line 23, through page 19, line 9 and original Claim 8);

60 weight percent to 85 weight percent aqueous liquid (*see, inter alia*, specification at page 20, lines 25-29);

0.01 weight percent to 10.0 weight percent additive, that is an adjuvant other than alum (*see, inter alia*, specification at page 7, lines 15-17, at page 20, lines 15-29 and original Claim 59); and

formulated with relative proportions of the liquid and the copolymer so that the copolymer interacts with the liquid to impart reverse thermal viscosity behavior to the composition, so that the viscosity of the composition increases when the temperature of the composition increases over some temperature range within 1°C and 37°C (*see, inter alia*, specification at page 16, lines 12-24 and at page, lines and original Claim 3.

Dependent Claims 4-7 each requires that the composition have properties with respect to gelation, with the composition being in the form of a flowable medium at a first, lower temperature and in the form of a gel at a second, higher temperature. (*See, inter alia*, specification at page 13, line 27 through page 14, line 8 and original Claim 4.) Claims 5-7 each recite additional specificity with respect to gelation, summarized as follows:

Claim 5: The first temperature is in a range of from 1°C to 20°C (*see, inter alia*, specification at page 16, lines 24-31 and original Claim 5);

Claim 6: The first temperature is in a range of from 1°C to 20°C and the second temperature is a range of from 25°C to 37°C (*see, inter alia*, specification at page 16, lines 24-31 and original Claim 6);

Claim 7: the copolymer is substantially all dissolved in the liquid at the first temperature, and at least a portion of the copolymer comes out of solution when the temperature is raised from the first temperature to the second temperature (*see, inter alia*, specification at page 8, lines 12-17, at page 14, lines 5-13 at page 24, lines 3-7 and original Claim 7).

Dependent Claims 9-14 each requires that the copolymer comprises at least one block each of a first polyoxyalkylene and a second polyoxyalkylene. (*See, inter alia*, specification at page 17, lines 23-26, and original Claim 9.) Claims 10-14 each recite additional specificity with respect to the copolymer, summarized as follows:

Claim 10: The first polyoxyalkylene is polyoxyethylene and the second polyoxyalkylene is polyoxypropylene (*see, inter alia*, specification at page 17, lines 8-13 at page 17, line 27 through page 18, line 13, and original Claim 10);

Claim 11: The requirements of Claim 10, wherein the copolymer has the formula $HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$, wherein a and each b are independently selected integers (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 11);

Claim 12: the formula of Claim 11, wherein the $(C_2H_4O)_b$ blocks together comprise at least 70 weight of the copolymer (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 12);

Claim 13: the formula of Claim 11, wherein a is between 15 and 80 and each b is independently between 50 and 150 (*see, inter alia*, specification at page 18, lines 19-33 and original Claim 13);

Claim 14: the copolymer has the formula $H(OCH_2CH_2)_b(OCHCH_2)_a(OCH_2CH_2)_bOH$ (with CH_3 on the $OCHCH_2$ group), wherein a is 20 to 80 and each b is independently 15 to 60 (*see, inter alia*, specification at page 18, lines 19-33, and original Claim 14).

Dependent Claims 15-31 each more particularly specifies the antigen (*See, inter alia*, specification at page 14, line 14 through page 15, line 12 and original Claims 15-31), with the subject matter of Claims 15-31 summarized as follows:

Claim 15: the antigen is selected from the group consisting of bacteria, protozoa, fungus, hookworm, virus and combinations thereof;

Claim 16: the antigen is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a non-pathogenic mutant of tetanus toxoid, a non-pathogenic mutant of diphtheria toxoid and combinations thereof;

Claim 17: the antigen is from Bordatella pertussis;

Claim 18: the antigen is from influenza virus;

Claim 19: the antigen is from M. tuberculosis;

Claim 20: the antigen immunizes against a childhood illness;

Claim 21: the antigen is from rotavirus;

Claim 22: the antigen is selected from the group consisting of a polysaccharide, a peptide mimetic of a polysaccharide, an antigen from Neisseria meningitiditis, an antigen from Streptococcus pneumoniae and combinations thereof;

Claim 23: the antigen is from Epstein-Barr virus;

Claim 24: the antigen is from Hepatitis C virus;

Claim 25: the antigen is from HIV;

Claim 26: the antigen comprises a molecule involved in a mammalian reproductive cycle;

Claim 27: the antigen is HCG;

Claim 28: the antigen is a tumor-specific antigen;

Claim 29: the antigen is from a blood-borne pathogen;

Claim 30: the antigen is a first antigen and the composition comprises a second antigen;

Claim 31: the limitations of Claim 30, and wherein the first antigen is selected from the group consisting of tetanus toxoid, a nonpathogenic mutant of tetanus toxoid and combinations thereof; and the second antigen is selected from the group consisting of diphtheria toxoid, a nonpathogenic mutant of diphtheria toxoid and combinations thereof.

Dependent Claims 33-37 each more particularly specifies the adjuvant (*see, inter alia*, specification at page 20, lines 7-14 and original claims 33-37), with the subject matter of Claims 33-37 summarized as follows:

Claim 33: the adjuvant comprises dimethyl dioctadecyl ammonium bromide (DDA);

Claim 34: the adjuvant comprises a CpG motif;

Claim 35: the adjuvant comprises a cytokine;

Claim 36: the adjuvant comprises chitosan material;

Claim 37: the adjuvant comprises N,O-carboxymethyl chitosan.

Dependent Claims 39-43 each requires the composition to be in a particular form or contained in a particular device. Dependent Claims 39 and 40 each requires that the composition is in the form of disperse droplets in a mist, with Claim 40 also requiring that the mist is produced by a nebulizer. Dependent Claims 41 and 42 each requires that the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the composition, with Claim 42 also requiring that the nebulizer is a nasal nebulizer. Dependent Claim 43 requires that the composition is contained within an injection device actuatable to administer the composition to the host by injection. (*See, inter alia*, specification at page 7, lines 8-14, at page 22, lines 20-23, at page 23, line 28 through page 24, line 2 and original Claims 39-43.)

Claim 44 is directed to a method of packaging and storing the composition of Claim 5, and requires placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, and wherein the gel form in the

container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container. (*See, inter alia*, specification at page 24, lines 8-18 and original Claim 44.)

Claim 148 requires that substantially all of the copolymer is dissolved in the liquid at some temperature within the temperature range recited in Claim 1, and Claim 149 requires that substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range recited in Claim 1. (*See, inter alia*, specification at page 7, lines 27-32, at page 10, lines 4-6, at page 14, lines 5-13 and at page 24, lines 3-5.)

Each of Claims 1, 4-7, 9-31, 33-37, 39-44, 148 and 149 is separately reviewable on appeal. None of the claims are to stand or fall together.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds for rejection in the Final Office Action are to be reviewed in this appeal:

Ground 1: Rejection of Claims 13, 14, 16-31, 33-37 and 39-42 without specification of a basis for rejection.

Ground 2: Rejection of Claims 1, 4-7, 9-12, 15, 43, 44, 148 and 149 under 35 U.S.C. § 102(b) as anticipated by Winter, A.J., Rowe, G.E., Duncan, J.R., Eis, M. J., Windom, J. Ganem, B. & Morein, B. Effectiveness of Natural and Synthetic Complexes of Porin and O Polysaccaride As Vaccines against *Brucella abortus* in Mice. *Infection and Immunity* 1988, Vol. 56, No. 11, pp. 2808-2817 (hereinafter, "Winter et al.") in light of the teaching of

Allison, A. Squalene and Squalane Emulsions as Adjuvants. *Methods* 1999, Vol. 19, pp. 87-93 (hereinafter, "Allison") and U.S. Patent No. 5,300,295 to Viegas et al. (hereinafter, "Viegas et al.").

ARGUMENT

The appealed claims involve a specific, but versatile composition for delivery of an antigen, in which the antigen is formulated with an adjuvant other than alum in a reverse thermal viscosity formulation, and in which a polyoxyalkylene block copolymer and an aqueous liquid interact to impart the reverse thermal viscosity behavior to the composition. Several example formulations are discussed in the application specification, including in the Examples section. Additional example formulations are discussed in a Rule 132 Declaration of Claire M. Coeshott submitted during prosecution, a copy of which is included in Appendix B. The rejections of claims made in the Final Office Action are not supportable, and it is respectfully requested that the rejections be reversed. Some specific deficiencies in the rejections at issue in this appeal are discussed below.

1. Ground 1

The rejection of Claims 13, 14, 16-31, 33-37 and 39-42 stated in the Final Office Action is not proper. Those claims are listed as being rejected on the Office Action Summary of the Final Office Action, without any basis for rejection being stated anywhere in the Final Office Action. Because no basis for rejection of those Claims has been stated, the rejection cannot stand.

2. Ground 2

The rejection of Claims 1, 4-7, 9-12, 15, 43, 44, 148 and 149 under 35 U.S.C. § 102(b) as anticipated by Winter et al. in light of the teaching of Allison and Viegas et al. is not proper. A copy of each of Winter et al., Allison and Viegas et al. is included in Appendix B.

On page 2 of the Final Office Action, in paragraph 4, the Examiner asserts that Winter et al. teach every limitation of Claim 1, citing to section (iii) in the first column of page 2810 of Winter et al., and the Examiner states:

> Winter et al. teaches a method for immunizing a mouse model comprising administering each mouse a 100 µl of a vaccine composition comprising 10 µg of antigen, 2.5 µg of reverse temperature copolymer L121 (5%), 10 µg/ adjuvant MDP (10%), 5 µl of sequalane [sic] and 95 µl of PBS-Tween . . . Because the proportions of major active components, which exhibit the enhanced immune response for the claimed composition including an antigen, copolymer and adjuvant, are all within the ranges of major active components as claim 1 drafted, the prior art anticipate the rejected claims. [Underlining added for emphasis.]

It appears as though the parenthesized percentage following each of L121 and MDP in the quoted portion of the Final Office Action refers to the Examiner's estimate of the percentage that each of these materials makes up of the total administration to each mouse. It is not clear, however, how these estimated percentages might have been determined.

The portion of Winter et al. cited by the Examiner (1st column of page 2810, section (iii)) states:

> The method of vaccine preparation for L-121 and MDP was kept as similar as possible to that used for TDM and MDP. bMDP solubilized in methanol was added to dried antigen in a glass homogenizer. After the methanol was completely evaporated, the pluronic polyol L-121 (BASF Wyandotte, Co., Parsippany, N.J.) and squalane were added and homogenized as previously described. A solution of PBS with 0.2% Tween 80 (PBS-Tween) was then added and emulsified with the mixture. Each mouse received 5 μg of antigen, 10 μg of bMDP, 2.5 μg of L-121, 5 μl of squalane, and 95 μl of PBS-Tween. [Citations to other references omitted.]

Based on this disclosure by Winter et al., the content of L-121 polymer in the administrative to each mouse is smaller than about 0.0026 weight percent (over 3 orders of magnitude smaller than the minimum of 5 weight percent of the polyoxyalkylene block copolymer required by Claim 1) and the content of PBS-Tween would be at least about 95 weight percent (significantly larger than the maximum of 85 weight of the aqueous liquid required by Claim 1). This inescapable conclusion is based on a simple recognition that the 95 μl of PBS-Tween specified by Winter et al. is an extremely large quantity in relation to the other components of the administration of Winter et al.

PBS is short for phosphate-buffered saline, as noted by Winter et al., second column of page 2808, and is mostly water. If the PBS-Tween is assumed conservatively for estimation

purposes to have a density equal to that of pure water (1 g/cc), then 95 μl of the PBS-Tween has a weight of about 95,000 μg ((95 μl PBS-Tween) x (1 g/cc) x (10^{-3} cc/μl) x (10^{6} μg/g) = 95,000 μg PBS-Tween). The 2.5 μg of L-121 polymer in the administration of Winter et al. equals only 0.0026 weight percent of the L-121 polymer relative to the PBS-Tween ((2.5/95,000)x100). And 0.0026 weight percent would be a high estimate of the concentration of the L-121 polymer in the administration of Winter et al., because the PBS-Tween would be expected to have a somewhat higher density than pure water due to dissolved solids, and the weights of the other components (antigen, bMDP and squalane) have not been included in calculating the 0.0026 weight percent value, so that the 0.0026 weight percent value is an upper limit estimate of the content, on a weight basis, of the L-121 polymer in the cited mouse administration of Winter et al.

Moreover, assuming that the squalane has a density equal to pure water (1 g/cc) for estimation purposes, then the weight of each component in, and the total weight of, the administration to each mouse according to the cited passage of Winter et al. would be as shown in Table A below.

Table A: Mouse Model From Winter et al., Page 2810

Component	Component Quantity Stated in Winter et al.	Component Weight, μg	Component Weight Percent
antigen	5 μg	5	0.0050
bMDP	10 μg	10	0.0100
L-121	2.5μg	2.5	0.0025
squalane	5μl	5,000*	4.9991
PBS-Tween	95 μl	95,000**	94.9834
Total		100,017.5	100.0000

*calculated, using density of 1 g/cc:
(5 μl squalane) x (1 g/cc) x (10^{-3} cc/μl) x (10^{6} μg/g) = 5,000 μg squalane
**calculated, using density of 1 g/cc:
(95 μl PBS-Tween) x (1 g/cc) x (10^{-3} cc/μl) x (10^{6} μg/g) = 95,000 μg PBS-Tween

As shown in Table A, the PBS-Tween makes up almost 95 weight percent of the administration to each mouse according to Winter et al. Also, this almost 95 weight percent estimate for the PBS-Tween is a lower limit estimate. Squalane, an oil, would be expected to have a density lower than that of pure water, meaning that the weight of squalane in the administration of Winter et al. would actually be smaller than the estimate shown in Table A, and the actual weight percentage of PBS-Tween would accordingly be even larger than shown in Table A. Also, as noted above, the PBS-Tween would be expected to have a density higher than that of pure water, meaning that the weight of the PBS-Tween would actually be larger than shown in Table A, and the actual weight percent of the PBS-Tween would accordingly be even higher.

Clearly, Winter et al. do not disclose the composition of Claim 1. The composition of Claim 1 requires, in combination with the other limitations, from 5 weight percent to 33

weight percent polyoxyalkylene block copolymer and from 60 weight percent to 85 weight percent aqueous liquid. Winter et al. clearly do not disclose a composition with those features. As discussed above and summarized in Table A, the portion on page 2810 of Winter et al. cited by the Examiner discloses an administration that includes L-121 polymer at a concentration that is over three orders of magnitude smaller than the minimum content of 5 weight percent of polyoxyalkylene block copolymer required by Claim 1 and that includes PBS-Tween at a concentration that is significantly larger than the maximum content of the aqueous liquid of 85 weight percent required by Claim 1. Viewed another way, Claim 1 recites a composition that is required to be comprised of at least 15 weight percent of components other than the aqueous liquid, whereas the administration given by Winter et al., as summarized in Table A, includes at most only about 5 weight percent of components other than the PBS-Tween solution. The mouse model administration disclosed in the portion of Winter et al. cited by the Examiner is clearly significantly different than the composition recited in Claim 1.

The Examiner does not assert that Allison or Viegas et al. disclose the claimed invention. A review of those references confirms that indeed neither Allison nor Viegas et al. discloses the claimed invention. Rather, the Examiner refers to Allison and Viegas et al. for what those references might teach in relation to the application of the teachings of Winter et al. to the claimed invention. The Final Office Action makes no statements as to the specific teachings of Allison or Viegas et al. A rejection based on Winter et al. in light of Allison and Viegas et al. was, however, made in a prior Office Action dated April 5, 2004 (hereafter, "4/5/04 Office Action"), in which the Examiner did make specific assertions with respect to Allison and Viegas et al. It is noted that the rejection based on Winter et al. in view of Allison and Viegas et al. in

the 4/5/04 Office Action was not made against the subject matter of the current Claim 1. In response to the 4/5/04 Office Action, compositional ranges for the antigen, polyoxyalkylene block copolymer, aqueous liquid and additive were added to Claim 1. The first time that the Examiner raised an issue based on Winter et al. in relation to the compositional ranges now in Claim 1 (previously in dependent Claim 38) was in the Final Office Action.

With respect to Viegas et al., on page 7 of the 4/5/04 Office Action, in paragraph 20, the Examiner states:

> Regarding to the limitation of the copolymer having a reverse thermal viscosity behavior, the copolymer of L121 inherently contains such characteristics in light of the teaching by Viegas et al. as described above.

The reference to "as described above", appears to be to a discussion in paragraph 17 of the 4/5/05 Office Action, beginning on page 6, in relation to a different rejection, where the Examiner states:

> Regarding to the limitation of the copolymer having a reverse thermal viscosity behavior, which causes the viscosity of the composition increases when the temperature of the composition increase over some temperature range from 1 °C to 37 °C, and the first temperature is the range of 1 °C to 20 °C, and the second temperature is in a range of 25 °C to 37 °C . . . Viegas et al. teach that the polypropylene/polyxyethylen [sic] block polymer formulated either as $HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$ or

> $H(OC_2H_2CH_2)_b(OCHCH_2)CH_{3a}(OC2H2CH2)_bOH$ is characterized as a heat sensitive polymer, in which the copolymer is in liquid form at room temperature or below and gel with desired osmolality at mammalian body temperature...

It is believed that the referenced formulas in the above quote are to formulas (IV) and (VI), respectively, presented in Column 4 of Viegas et al. In that regard, it is noted that formula (VI) in Viegas is stated as a specific formula (with a=49 and b=67, using the Examiner's notation). These polymers, as with other polymers disclosed by Viegas et al., contain a large weight percentage of polyoxyethylene. Generally, Viegas et al. specify the use of polyoxyalkylene block copolymers with "polyoxyethylene moiety constituting at least 60% by weight of the copolymer." *See*,Viegas et al., at column 5, lines 53-64. Viegas et al. further disclose that polymer of formula (IV) contains at least 70% by weight of polyoxyethylene (*see*, Viegas et al., at column 6, lines 15-23). Moreover, the formula (VI) polymer of Viegas et al. contains approximately 52.5 weight percent polyoxyethylene, based on the relative molecular weights of the oxyethylene repeating units (44) and the oxypropylene repeating units (58), and the number of the oxyethylene repeating units (98) and the oxypropylene repeating units (67) in the polymer. Viegas et al. also specify that copolymers are present, "preferably, in the amount of about 10 to about 30% by weight of the total weight of the compositions of the invention." *See*,Viegas et al., at column 6, lines 43-45.

Conversely, the noted Pluronic L121 polymer disclosed in Winter et al., contains a much smaller content of polyoxyethylene than the noted polymers of formula (IV) or (VI) of Viegas et al. Referring to Allison, it is disclosed that "Pluronic L121 (also termed poloxamer 401)

contains 10 weight percent of POE [polyoxyethylene]..." *See*, Allison, 1st column on page 89, under the heading "Nonionic Block Copolymers." Respectfully, it is not appreciated how the disclosure by Viegas et al., with respect to polymers with high polyoxyethylene content at a concentration of about 10 to 30% by weight, sheds any light on inherent properties of the cited mouse administration disclosed by Winter et al., discussed above, containing the Pluronic L121 polymer, which has a very low polyoxyethylene content and is used at a much lower concentration (less than 0.0026 weight percent, as discussed above). The Examiner's assertion with respect to the inherent properties of the administration in Winter et al. in light of Viegas et al. is simply not supported by the disclosure of Viegas et al.

With respect to Allison, on page 6 of the 4/5/04 Office Action, in paragraph 16, the Examiner states:

> Allison teaches that squalane or squalane emulsion are efficient adjuvants, eliciting both human and cellular immune response . . . Allison particularly points out that the triblock copolymer, such as Pluronic L121, added to squalane emulsions augments their adjuvant effect.

The Examiner makes this reference apparently for the proposition that squalane or squalane emulsion, such as is used in the administration of Winter et al., is an adjuvant. But, as noted above, Winter et al. is clearly deficient in other regards, notwithstanding the teaching of Allison.

It is clear that Winter et al., whether or not in light of the teaching of Allison and Viegas et al., do not anticipate the subject matter of Claim 1 or of any rejected dependent claim. Moreover, the following comments are made in relation to specific rejected dependent claims.

Dependent Claim 148

Winter et al do not disclose the combination of features disclosed in dependent Claim 148. Moreover, Allison contains a specific teaching away that Winter et al. anticipate the subject matter of pending Claim 148. In the 1st column of page 89 of Allison, under the heading "Nonionic Block Copolymers", there is a discussion concerning the L-121 polymer in relation to squalane emulsions. In particular Allison states:

> Thus L121 is poorly soluble in water, does not stabilize emulsions and is termed a spreading agent. It adheres to the surface of the oil droplets in aqueous media. [Underlining added for emphasis.]

Dependent Claim 148 requires that substantially all of the polyoxyalkylene block copolymer is dissolved in the aqueous liquid at least when the claimed composition is at some temperature within the temperature range stated in Claim 1. The portion of Allison cited above teaches that the L121 polymer in a squalane emulsion adheres to the oil phase (i.e., the squalane) and thus does not dissolve in the aqueous phase, and this is a teaching away that the squalane-containing vaccine preparation disclosed in the 1st column, page 2810, section (iii) of Winter et al. would include the features of Claim 148.

Dependent Claim 149

Dependent Claim 149 requires that substantially all of the polyoxyalkylene block copolymer and the antigen are dissolved in the aqueous liquid at least when the claimed composition is at some temperature within the temperature range stated in Claim 1. Winter et al. do not disclose this combination of elements of Claim 149. Moreover, as discussed above with respect to dependent Claim 148, Allison provides a specific teaching away that Winter et al. anticipate the subject matter of Claim 148, and particularly with regard to the requirement of Claim 149 that substantially all of the polyoxyalkylene block copolymer is dissolved in the aqueous liquid at some temperature within the recited temperature range.

Dependent Claim 4

Dependent Claim 4 requires that the claimed composition is in the form of a flowable medium when the composition is at a first temperature in the temperature range recited in Claim 1, and that the claimed composition is in the form of a gel when the composition is at a second, higher temperature within that temperature range. Winter et al. do not disclose such a composition, and there is no reasonable basis for concluding that the mouse administration cited to by the Examiner would inherently include such properties, especially considering the minute amount of polymer present in the mouse administration of Winter et al. and the nature of the polymer used by Winter et al. as adhering to the squalane phase, as discussed above.

Dependent Claim 5

In addition to the requirements of Claim 4, Claim 5 requires that the first temperature is in a range of from 1 °C to 20 °C. In addition to not disclosing the subject matter of Claim 4, Winter et al. also do not disclose this additional subject matter of Claim 5, and there is no reasonable basis for concluding that the mouse administration cited to by the Examiner would inherently include such properties, which are even more specific than the properties discussed above with respect to Claim 4.

Dependent Claim 6

In addition to the requirements of Claim 4, Claim 6 requires that the first temperature is in a range of from 1 °C to 20 °C and the second temperature is in a range of form 25 °C and 37 °C. In addition to not disclosing the subject matter of Claim 4, Winter et al. also do not disclose this additional subject matter of Claim 6, and there is no reasonable basis for concluding that the mouse administration cited to by the Examiner would inherently include such properties, which are even more specific than the properties discussed above with respect to Claim 4.

Dependent Claim 7

In addition to the requirements of Claim 4, Claim 7 requires that the copolymer is substantially all dissolved in the liquid when the composition is at the first temperature, and at least a portion of the copolymer comes out of solution in the liquid when the temperature of the composition is raised from the first temperature to the second temperature. In addition to not disclosing the subject matter of Claim 4, Winter et al. also do not disclose this additional subject matter of Claim 7, and there is no reasonable basis for concluding that the mouse administration

cited to by the Examiner would inherently include such properties, which are even more specific than the properties discussed above with respect to Claim 4. Moreover, as discussed above with respect to dependent Claims 148 and 149, Allison provides a specific teaching away that Winter et al. anticipate the subject matter of Claim 7, and particularly with regard to the requirement of Claim 7 that substantially all of the copolymer is dissolved in the aqueous liquid at the first temperature and that at least a portion of the copolymer comes out of solution when the temperature is raised from the first temperature to the second temperature.

Dependent Claim 12

Dependent Claim 12 requires the polyoxyalkylene block copolymer to have a particular formula with at least 70 weight percent of specified $(C_2H_4O)_b$ blocks, or significantly more than the 10 weight percent polyoxyethylene content of the L-121 polymer disclosed in the portion of Winter et al. cited by the Examiner, as discussed above.

Dependent Claim 43

Dependent Claim 43 requires the composition to be contained within an injection device. Winter et al. do not disclose such a composition contained within an injection device.

Dependent Claim 44

Dependent Claim 44 requires placing the composition in a container when the composition is in the form of a flowable medium and thereafter raising the temperature of the

composition in the container to convert the composition to a gel form. Winter et al. do not disclose the claimed subject matter including these additional features stated in Claim 44.

CONCLUSION

The rejections stated in the Final Office Action are unsupportable, and it is respectfully requested that the rejections be reversed, and the application proceed to issuance.

Respectfully submitted,

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APPENDIX A
CLAIMS

1. (Appealed) A composition for delivery of an antigen for stimulation of an immune response when administered to a host, the composition comprising:

an antigen, a polyoxyalkylene block copolymer and an aqueous liquid;

the polyoxyalkylene block copolymer being biocompatible, not having toxic or injurious effects on biological function in the host when the composition is administered;

wherein, the composition is formulated with relative proportions of the liquid and the copolymer so that the copolymer interacts with the liquid to impart reverse thermal viscosity behavior to the composition, so that the viscosity of the composition increases when the temperature of the composition increases over some temperature range within 1 °C to 37 °C; and

wherein, the composition further comprises an additive enhancing the immune response when the composition is administered to the host, the additive being an adjuvant other than alum; and;

wherein, the liquid comprises from 60 weight percent to 85 weight percent of the composition, the antigen comprises from 0.0001 weight percent to 5 weight percent of the composition, the copolymer comprises from 5 weight percent to 33 weight percent of the composition and the additive comprises from 0.01 weight percent to 10.0 weight percent of the composition.

2. (Cancelled)

3. (Cancelled)

4. (Appealed) The composition of Claim 1, wherein the composition is in the form of a flowable medium when the composition is at a first temperature in the temperature range and

the composition is in a gel form when the composition is at a second temperature in the temperature range, the second temperature being higher than the first temperature.

5. (Appealed) The composition of Claim 4, wherein the first temperature is in a range of from 1 °C to 20 °C.

6. (Appealed) The composition of Claim 4, wherein the first temperature is in a range of from 1 °C to 20 °C and the second temperature is in a range of from 25° C to 37 °C.

7. (Appealed) The composition of Claim 4, wherein the copolymer is substantially all dissolved in the liquid when the composition is at the first temperature, and at least a portion of the copolymer comes out of solution in the liquid when the temperature of the composition is raised from the first temperature to the second temperature.

8. (Cancelled)

9. (Appealed) The composition of Claim 1, wherein the polyoxyalkylene block copolymer comprises at least one block of a first polyoxyalkylene and at least one block of a second polyoxyalkylene.

10. (Appealed) The composition of Claim 9 wherein the first polyoxyalkylene is polyoxyethylene and the second polyoxyalkylene is polyoxypropylene.

11. (Appealed) The composition of Claim 10, wherein the polyoxyalkylene block copolymer has the formula:

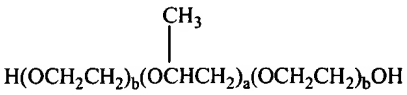
$HO(C_2H_4O)_b(C_3H_6O)_a(C_2H_4O)_bH$

wherein a and each b are independently selected integers.

12. (Appealed) The composition of Claim 11, wherein the $(C_2H_4O)_b$ blocks together comprise at least 70 weight percent of the polyoxyalkylene block copolymer.

13. (Appealed) The composition of Claim 11 wherein a is between 15 and 80 and each b is independently between 50 and 150.

14. (Appealed) The composition of claim 10, wherein the polyoxyalkylene block copolymer has the formula:



wherein a is 20 to 80 and each b is independently 15 to 60.

15. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of bacteria, protozoa, fungus, hookworm, virus and combinations thereof.

16. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a non-pathogenic mutant of tetanus toxoid, a non-pathogenic mutant of diphtheria toxoid and combinations thereof.

17. (Appealed) The composition of Claim 1, wherein the antigen is from Bordatella pertussis.

18. (Appealed) The composition of Claim 1, wherein the antigen is from influenza virus.

19. (Appealed) The composition of Claim 1, wherein the antigen is from M. tuberculosis.

20. (Appealed) The composition of Claim 1, wherein the antigen immunizes against a childhood illness.

21. (Appealed) The composition of Claim 1, wherein the antigen is from rotavirus.

22. (Appealed) The composition of Claim 1, wherein the antigen is selected from the group consisting of a polysaccharide, a peptide mimetic of a polysaccharide, an antigen from Neisseria meningitiditis, an antigen from Streptococcus pneumoniae and combinations thereof.

23. (Appealed) The composition of Claim 1, wherein the antigen is from Epstein-Barr virus.

24. (Appealed) The composition of Claim 1, wherein the antigen is from Hepatitis C virus.

25. (Appealed) The composition of Claim 1, wherein the antigen is from HIV.

26. (Appealed) The composition of Claim 1, wherein the antigen comprises a molecule involved in a mammalian reproductive cycle.

27. (Appealed) The composition of Claim 1, wherein the antigen is HCG.

28. (Appealed) The composition of Claim 1, wherein the antigen is a tumor-specific antigen.

29. (Appealed) The composition of Claim 1, wherein the antigen is from a blood-borne pathogen.

30. (Appealed) The composition of Claim 1, wherein the antigen is a first antigen and the composition comprises a second antigen.

31. (Appealed) The composition of Claim 30, wherein the first antigen is selected from the group consisting of tetanus toxoid, a nonpathogenic mutant of tetanus toxoid and combinations thereof; and

the second antigen is selected from the group consisting of diphtheria toxoid, a nonpathogenic mutant of diphtheria toxoid and combinations thereof.

32. (Cancelled)

33. (Appealed) The composition of claim 1, wherein the adjuvant comprises dimethyl dioctadecyl ammonium bromide (DDA).

34. (Appealed) The composition of Claim 1, wherein the adjuvant comprises a CpG motif.

35. (Appealed) The composition of Claim 1, wherein the adjuvant comprises a cytokine.

36. (Appealed) The composition of claim 1, wherein the adjuvant comprises chitosan material.

37. (Appealed) The composition of claim 36, wherein the adjuvant comprises N,O-carboxymethyl chitosan.

38. (Cancelled)

39. (Appealed) The composition of Claim 1, wherein the composition is in the form of disperse droplets in a mist.

40. (Appealed) The composition of Claim 39, wherein the mist is produced by a nebulizer.

41. (Appealed) The composition of Claim 1, wherein the composition is contained within a nebulizer actuatable to produce a mist comprising dispersed droplets of the composition.

42. (Appealed) The composition of Claim 40, wherein the nebulizer is a nasal nebulizer.

43. (Appealed) The composition of claim 1, wherein the composition is contained within an injection device that is actuatable to administer the composition to the host by injection.

44. (Appealed) A method of packaging and storing the composition of claim 5, comprising placing the composition in a container when the composition is in the form of a flowable medium and, after the placing, raising the temperature of the composition in the container to convert the composition to the gel form for storage, wherein the gel form in the container can be converted back to the form of a flowable medium for administration to the host by lowering the temperature of the composition in the container.

45-147 (Cancelled)

148. (Appealed) The method of Claim 1, wherein substantially all of the copolymer is dissolved in the liquid at some temperature within the temperature range.

149. (Appealed) The method of Claim 1, wherein substantially all of the copolymer and the antigen are dissolved in the liquid at some temperature within the temperature range.

150-197. (Withdrawn)

APPENDIX B
EVIDENCE

1. Rule 132 Declaration of Claire M. Coeshott, dated April 22, 2003, including Appendices; acknowledged by the Examiner in an Office Action dated July 15, 2003, paragraph 1, and in an Office Action dated April 5, 2004, paragraph 1.

2. Winter, A.J., Rowe, G.E., Duncan, J.R., Eis, M. J., Windom, J.. Ganem, B. & Morein, B. Effectiveness of Natural and Synthetic Complexes of Porin and O Polysaccaride As Vaccines against *Brucella abortus* in Mice. *Infection and Immunity* 1988, Vol. 56, No. 11, pp. 2808-2817; entered by the Examiner on a Notice of References Cited (PTO-892) accompanying an Office Action dated April 5, 2004.

3. Allison, A. Squalene and Squalane Emulsions as Adjuvants. *Methods* 1999, Vol. 19, pp. 87-93; entered by the Examiner on a Notice of References Cited (PTO-892) accompanying an Office Action dated April 5, 2004.

4. U.S. Patent No. 5,300,295 to Viegas et al.; entered by the Examiner on a Notice of References Cited (PTO-892) accompanying an Office Action dated April 5, 2004.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit 1648
BLONDER et al.	Examiner: Li, Bao Q.
Serial No.: 09/888,235	RULE 132 DECLARATION OF CLAIRE M. COESHOTT (37 C.F.R. § 1.132)
Filed: June 22, 2001	
Atty. File No.: 42830-00234	
For: "DELIVERY VEHICLE COMPOSITION AND METHODS FOR DELIVERING ANTIGENS AND OTHER DRUGS"	

CERTIFICATE OF MAILING

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231 ON April 22, 2005.

MARSH FISCHMANN & BREYFOGLE, LLP

BY: [signature]

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Madam:

Claire M. Coeshott, residing at 875 South Josephine Street, Denver, Colorado 80209 80027, declare as follows:

I am currently employed in the capacity of Director, Vaccine Technologies by RxKinetix, Inc., the assignee of the referenced U.S. Patent Application.

The attached Exhibit A is a summary of my technical qualifications.

The attached Exhibit B summarizes some tests (identified as Examples 11-16 for convenient reference) performed by me or by others at my direction concerning compositions for delivery of antigens. Examples 11-16 presented in Exhibit B concern formulation and testing of antigen delivery test compositions in which the antigen is formulated in an aqueous liquid with an adjuvant material and a polymer of a type and in an amount to impart reverse-thermal viscosity behavior to the composition. Antigens subject to the testing include tetanus toxoid (TT), diphtheria toxoid (DT) and recombinant anthrax protective antigen (rPA); adjuvant materials tested include those containing chitosan or CpG dinucleotide motifs (CpG); and the polymer for all tests is Pluronic® F127 polymer. Studies in mice compared the performance of these test compositions as compared to comparison compositions in which the antigen is differently formulated. Results of these mice studies are

discussed in Exhibit B, with tabular results of mice antibody response data for Examples 12-15 being presented in attached Exhibits C-F. The results of the mice studies presented in Exhibits B-F demonstrate a high level of antibody response to the test composition, and with the antibody response to the test composition most often being both faster to develop and attaining a higher level than the antibody response to the comparison compositions, as indicated by antibody assays. The attainment of a higher level of antibody response is obviously important. Perhaps more important, however, is the faster antibody response to the test compositions. In a high-risk situation, such as an epidemic, development of quicker immunization response following antigen administration may mean the difference between someone surviving or not surviving the situation. This more rapid response to immunization is surprising as it might be expected that administering the antigen in the reverse-thermal viscosity composition would delay distribution of the antigen to the relevant cells of the immune system, thus slowing any immune response.

Example 11 presents a general procedure for preparing formulations and for performing and obtaining antibody assays to determine antibody response. Examples 12-16 discuss preparation of specific formulations and mice studies on those particular formulations, generally as described in Example 11 except as noted.

In Example 12, test compositions with TT are formulated with 16.25% (w/w) Pluronic®F127 polymer and with varying amounts of an adjuvant material containing chitosan (0.5, 0.17, or 0.05% (w/w) of the adjuvant material). Comparative compositions with TT are also formulated with only the adjuvant material or with only the polymer. The test compositions demonstrate higher IgG antibody response at both two weeks and at five weeks following a single subcutaneous administration of 0.5 Lf TT than the comparable comparative compositions, as clearly summarized in the following table, which provides data for geometric mean and average IgG antibody titers in serum samples from the mice studies on the different compositions.

Chitosan Adjuvant Material Content	IgG Antibody Titers – Geometric Mean and (Average)		
	Test Comp. With Both Adj. Mtl. & Polymer	Comparative Comp. With Only Adj. Mtl.	Comparative Comp. With Only Polymer
Two Weeks Following Administration			
0.5% (w/w)	413 (445)	162 (396)	
0.17% (w/w)	497 (665)	337 (467)	
0.05% (w/w)	252 (271)	215 (236)	
0% (w/w)			27 (56)
Five Weeks Following Administration			
0.5% (w/w)	14,132 (18,836)	4748 (5403)	
0.17% (w/w)	11,201 (13,194)	9,119 (11,442)	
0.05% (w/w)	5,437 (7,055)	4,862 (6,165	
0% (w/w)			122 (289)

As summarized in the above table, the comparative composition formulated with only Pluronic® F127 polymer, and no adjuvant material, performed poorly. Comparative compositions formulated with only the adjuvant material, and no Pluronic® F127 polymer performed better than comparative compositions formulated with only Pluronic® F127 polymer, but the test compositions, formulated with both the adjuvant material and the Pluronic® F127 polymer, performed the best.

In Example 13, test compositions with TT are formulated with 16.25% (w/w) Pluronic® F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with TT are also formulated without the Pluronic® F127 polymer, but with the CpG-containing adjuvant material with and without the addition also of glycerol or incomplete Freund's adjuvant (IFA). The test compositions demonstrate higher IgG antibody response following a single subcutaneous administration of 0.5 Lf TT than the comparative compositions. It is of particular interest to point out that IFA is considered a "gold standard" for adjuvants used in immunization of experimental animals and that the test composition is an improvement. In Example 14, test compositions with TT are formulated with 16.25% (w/w) Pluronic® F127 polymer and with various amounts of an adjuvant material containing CpG (20, 6.7 or 2 % (v/w) of the adjuvant material). Comparative

compositions with TT are also formulated with only the adjuvant material or with only the polymer. The test compositions consistently demonstrate higher IgG antibody response at two, four and eight weeks following a single subcutaneous administration of 0.5 Lf TT than the comparable comparative compositions, as clearly summarized in the following table, which provides data for geometric mean and average IgG antibody titers in serum samples from the mice studies on the different compositions.

CpG Adjuvant Material Content	IgG Antibody Titers – Geometric Mean and (Average)		
	Test Comp. With Both Adj. Mtl. & Polymer	Comparative Comp. With Only Adj. Mtl.	Comparative Comp. With Only Polymer
Two Weeks Following Administration			
20%(v/w)	6,974 (7,705)	5,287 (5,792)	
6.7% (v/w)	1,761 (1,969)	476 (554)	
2% (v/w)	694 (792)	264 (284)	
0% (vw)			623 (780)
Four Weeks Following Administration			
20%(v/w)	14,768 (32,636)	6,050 (8,309)	
6.7% (v/w)	77,632 (101,667)	3,225 (3,472)	
2% (v/w)	14,037 (18,054)	2,243 (2,282)	
0% (v/w)			626 (884)
Eight Weeks Following Administration			
20%(v/w)	39,903(77,778)	14,429(46,467)	
6.7% (v/w)	76,792(172,083)	8,566(11,619)	
2% (v/w)	17,065(27,739)	4,034(4,714)	
0% (v/w)			345(926)

As summarized in the above table, the comparative composition formulated with only Pluronic® F127 polymer, and no adjuvant material, performed poorly. Comparative compositions formulated with only the adjuvant material, and no Pluronic® F127 polymer performed better than comparative compositions formulated with only Pluronic® F127 polymer, but the test compositions, formulated with both the adjuvant material and the Pluronic®F127 polymer, consistently performed the best. The

values for the 20% test composition may be lower than expected in this example due to technical difficulties performing the assay.

In Example 15, test compositions with DT are formulated with 16.25% (w/w) Pluronic®F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with DT are also formulated without the Pluronic® F127 polymer, but with the adjuvant material containing CpG. The test compositions demonstrate attainment of a higher IgG antibody response following a single subcutaneous administration of 1 Lf DT than the comparative compositions, although at later times (after 12 weeks) following administration, the comparative compositions do result in similiar IgG antibody responses.

In Example 16, test compositions with rPA are formulated with 16.25% (w/w) Pluronic® F127 polymer and with 20% (v/w) adjuvant material containing CpG. Comparative compositions with rPA are also formulated without the Pluronic® F127 polymer, but with either the CpG-containing adjuvant material or alternatively with aluminum hydroxide (alum). The test compositions demonstrate attainment of a higher IgG antibody response following a single subcutaneous administration of 25 μg rPA than the comparative compositions including the alum. Also, the test compositions resulted in significantly higher toxin neutralization antibody titers than either the comparison compositions with the CpG-containing adjuvant or the comparison compositions containing alum. The toxin neutralization assay is a measure of the ability of the test composition to raise an antibody response that protects cells against challenge with anthrax lethal toxin and therefore is an excellent indicator of the effectiveness of the test composition.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of this patent application or any patent issuing thereon.

Respectfully submitted,

Date: 4/22/03

By: C M Coeshott

Claire M. Coeshott

EXHIBIT A
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

BIOGRAPHICAL SKETCH AND TECHNICAL QUALIFICATIONS

NAME Coeshott, Claire M.	TITLE Director, Vaccine Technologies

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
University of Bristol, England	B.Sc., 1st class, honors	1978	Pathology
University of Bristol, England	Ph.D.	1982	Immunology

RESEARCH AND PROFESSIONAL EXPERIENCE:

Employment

1981-1982	Research Assistant, Department of Pathology, University of Bristol, England.
1982-1985	Research Fellow, National Jewish Hospital and Research Center, Denver, Colorado.
1985-1988	Research Associate, Division of Membrane Biology, Medical Biology Institute, La Jolla, California.
1988-1994	Group Leader: Immunology, Cortech Inc., Denver, Colorado.
1991-1994	Team Leader: Lupus Project, Cortech Inc., Denver, Colorado.
1994-1996	Group Leader: Immunoassay Methods, Biopharmaceutics Department, Cortech Inc., Denver, Colorado.
1996-1997	Group Leader: Protease Inhibitor Program, Pharmacology Department, Cortech Inc., Denver, Colorado.
1997-1998	Research Fellow and Group Leader, Biology Department, Ribozyme Pharmaceuticals Inc., Boulder, Colorado.
1999-2000	Senior Scientist, Ceres Pharmaceuticals Ltd., Denver, Colorado.
2000-2002	Senior Scientist, RxKinetix Inc. Louisville, Colorado.
2002	Director, Vaccine Technologies, RxKinetix Inc. Louisville, Colorado.

Synopsis Of Industrial Experience

RxKinetix Inc. 2000 – present
Project leader for vaccine program to evaluate proprietary formulations for vaccine delivery. Coordinate research effort in house and with outside collaborators. Develop

assays for measurement of antibody and T cell responses to formulations. Liaise with business development and legal departments for optimal positioning of technology.

Globeimmune, Inc (formerly Ceres Pharmaceuticals Ltd.) 1999 – 2000
Employed as both bench scientist and manager for a SBIR-funded project to develop a genetically-engineered microorganism as an HIV vaccine. Designed and executed *in vivo* and *in vitro* experiments for vaccine program:

- obtained Proof of Principle for vaccine candidate using a tumor protection model in mice.

Ribozyme Pharmaceuticals Inc. 1997 – 1998
Led multidisciplinary project to develop ribozyme-based therapeutic to treat chemoresistance in cancer. Team consisted of 3 Ph.Ds. and 4 RAs. In addition, was line manager for 3 RAs within Biology Group:

- co-ordinated synthesis of ribozymes, designed in vitro experiments with RNA endpoints (RNase protection assay and Taqman analysis) and phenotypic endpoints (apoptosis).
- designed and oversaw in vivo experiments to test lead compounds using human cancer cell line xenografts in athymic mice

Cortech Inc. 1989 – 1997
Immunology Program: Basic Research
Developed Immunology program using multivalent arrays of haptens on large molecular weight carriers such as dextran to suppress or stimulate hapten-specific antibody responses in mice. Outcomes of program:

- patent issued (November 1996) addressing stimulatory aspects of technology which formed basis of vaccine program at Cortech.
- filing of an IND application (March 1995) and subsequent completion of phase I clinical trial for a specific immunomodulator, CI-0694, to suppress sulfamethoxazole hypersensitivity in AIDS patients .

Set up tissue culture laboratory as service facility for providing monoclonal antibodies to other projects:

- developed and characterized peptide-specific helper T cell hybridomas and their responses to various Cortech compounds.
- demonstrated activity of Cortech compounds for cytotoxic T cell induction.
- developed and characterized monoclonal antibodies against fibrinopeptides and bradykinin antagonists.

Protease Inhibitor Program: Research
Program addressed potential of novel synthetic, substrate-based compounds to inhibit enzymatic degradation of tissues and release of various cytokines:

- designed and executed assays for measuring impact of inhibitors on cytokine production (TNFα, IL-1β, IL-2, IL-8) from whole blood as well as from various cell types including THP-1 monocytic cell line, Jurkat, neutrophils and monocytes isolated from human peripheral blood.
- oversaw development of extracellular matrix assay to test inhibition of radiolabelled matrix degradation.

Managerial

As leader of Lupus project, coordinated a team of up to three Ph.Ds. and four RAs in the production of compound to suppress nephritis occurring in the autoimmune disease, systemic lupus erythematosus:

- initiated and oversaw collaborations with researchers in field to assess recognition of Cortech compounds by antibodies from human SLE patients.
- developed ELISPOT assay to measure anti-DNA and anti-histone antibody-secreting cells.
- designed and executed all in vivo experiments to monitor the effects of these constructs in lupus-prone mice.
- lead compound identified.

Pre-clinical Research

As member of Biopharmaceutics department, supervised two senior- level RAs and one post-doctoral researcher:

- developed immunoassays to measure specific antibody responses in AIDS patients entering phase I clinical trial of CI-0694. ELISA and competition ELISA for IgM, IgA and IgG developed and subsequently used for measurement of antibodies in samples from phase I trial. Liased with AIDS Clinical Trial Group (ACTG) in evaluation of CI-0694.
- collaborated with physicians at Denver General Hospital in study to investigate correlation between antibody levels and failure of desensitization to sulfamethoxazole.
- coordinated clinical studies to examine efficacy of elastase inhibitor, CE-1037, in cystic fibrosis and ARDS: defined sample handling procedures for BALF and sputum; participated in site visits and initiation of two clinical trials.
- wrote research reports and SOPs; reviewed INDs, clinical protocols and other documents.

Awards, Honors, Grants

1. Leukemia Society of America Special Fellowship, July 1987 - July 1990.
2. University of Bristol Postgraduate Scholarship, 1978 - 1981.

Memberships

British Society for Immunology

Patents

1 issued; 2 applications

Selected Publications

Grace S.A., Elson, C.J. and Coeshott, C.M. Production of anti-host IgG by transfer of primed histocompatible cells. **Clin. Exp. Immunol. 39:449, 1980.**

Elson, C.J. and Coeshott, C.M. Tolerance of allotypic determinants induced by lymphoid cells from congenic mice bearing the allotype. **Immunol. 43:281, 1981.**

Coeshott, C.M. and Grey, H.M. Transfer of antigen presenting capacity to la negative cells upon fusion with la-bearing liposomes. **J. Immunol. 134:1343, 1985.**

Gay, D., Coeshott, C.M., Golde, W., Kappler, J. and Marrack, P. The Major Histocompatibility Complex-restricted antigen receptor on T cells IX. Role of accessory molecules in recognition of antigen plus isolated IA. **J. Immunol. 136:2026, 1986.**

Coeshott, C.M., Chesnut, R.W., Kubo, R.T., Grammer, S.F., Jenis, D.M. and Grey, H.M. Ia-specific mixed leukocyte reactive T cell hybridomas: Analysis of their specificity by using purified class II MHC molecules in a synthetic membrane system. **J. Immunol. 136:2832, 1986.**

Blodgett, J.K., Coeshott, C.M., Roper, E.F., Ohnemus, C., Allen, L.G., Kotzin, B.L. and Cheronis, J.C. Synthesis and characterization of novel antigen-specific immunosuppressive agents and their utilization in the (NZB x NZW)F1 murine model of systemic lupus erythematosus. **Proc. Amer. Pep. Symp. 12: 873, 1992.**

Coeshott, C., Allen, L., McLeod, D., Cheronis, J. and Kotzin, B. Antigen-specific suppression of antibody responses: implications for vaccine design. **Vaccines 95. Cold Spring Harbor Laboratory Press, 1995.**

De la Cruz, V.F., Cook, C., Allen, L., Strong, P., Blodgett, J., Ohnemus, C., McCall, C., Goodfellow, V., McLeod, D., Gross, K., Cheronis, J. and Coeshott, C. Antigen-specific Immunomodulation (ASIM): the rational design of molecules that are inherently immunogenic. **Vaccines 95. Cold Spring Harbor Laboratory Press, 1995.**

Pilyavskaya, A., Wieczorek, M., Asztalos, J., Coeshott, C., Francis, M.D. and Blodgett, J. Purification of $F(ab')_2$ and Fab' fragments from the T cell receptor-specific monoclonal antibodies, F23.1 and KJ16, and preparation of conjugates with dexamine. **J. International Bio-chromatography, 3: 215, 1996.**

Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S.E., Wieczorek, M. Kroona, H., Leimer, A. and Cheronis, J. Converting enzyme-independent release of TNFα and IL-1β from stimulated THP-1, a human monocytic cell line, in the presence of activated neutrophils or purified proteinase 3. **Proc. Natl. Acad. Sci. USA, 96: 6261, 1999.**

Stubbs, A.C., Martin, K.S., Coeshott, C., Skaates, S.V., Kuritzkes, D.R., Bellgrau, D., Franzusoff, A., Duke, R.C. and Wilson, C.C. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. **Nature Medicine 7:625-629, 2001.**

Westerink, M.A.J., Smithson, S.L., Srivastava, N., Blonder, J., Coeshott, C., and Rosenthal, G.J. Projuvant™ (Pluronic F127®/chitosan) enhances the immune response to intranasally administered tetanus toxoid. **Vaccine 20: 711-723, 2001.**

EXHIBIT B
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 11: General procedure for preparing and testing antigen delivery compositions

Preparation of formulations: Pluronic® F127 polymer (National Formulary pharmaceutical grade, BASF, Washington, NJ) stock solution was prepared at 34% (w/w) by dissolving in ice-cold PBS with complete dissolution achieved by storing overnight (ON) at 4°C. Protasan® (Chitosan chloride, ultrapure CL 213; Pronova Biomedical, Oslo, Norway; MW = 272,000; 84% deacetylated) stock solutions were prepared at 3% (w/w) in 1.0 % (v/v) acetic acid in sterile water (USP grade) and were heated at 37°C to dissolve. An adjuvant containing CpG dinucleotide motifs (CpG) was obtained from Qiagen (ImmunEasy™, proprietary formulation containing CpG of Qiagen Inc. Valencia, CA) and was added to formulations according to the manufacturer's instructions. The antigens evaluated include recombinant anthrax protective antigen (rPA), tetanus toxoid (TT), and diphtheria toxoid (DT). Adjuvants, such as those containing chitosan or CpG, were also added to the formulations. Unless otherwise noted, the stock solutions were mixed together to prepare formulations containing various combinations of antigen, adjuvant and Pluronic® F127 polymer.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN) 6 to 8 weeks of age were used for these studies. Groups of mice were immunized once subcutaneously (s.c.) with antigens in various formulations on day 0.

Antibody assays: The serum antibody responses to antigens were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with the appropriate concentration of antigen in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Serum samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing tetramethylbenzidine (TMB) (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Statistics: Data were analyzed for differences using Students t test. A probability (p) of 0.05 or less was considered significant. Outliers were identified by Grubb's test.

Testing of performance of specific formulations with antigens TT, DT and rPA are discussed below in Examples 12-16.

EXAMPLE 12: TT with chitosan-containing adjuvant in the composition

Preparation of formulations: TT and Pluronic® F127 stock solutions were prepared as described in Example 1. Protasan® stock solution was prepared at 3% (w/w) in 1.0 % (v/v) acetic acid in sterile water (USP grade). Tetanus toxoid (Accurate Chemical & Scientific, Westbury, NY)

contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5 Lf/ml), 0.5% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml), 0.17% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(iii) TT (5 Lf/ml), 0.05% (w/w) chitosan and 16.25% (w/w) Pluronic® F127;
(iv) TT (5 Lf/ml) and 0.5% (w/w) chitosan (no Pluronic® F127);
(v) TT (5 Lf/ml) and 0.17% (w/w) chitosan (no Pluronic® F127);
(vi) TT (5 Lf/ml) and 0.05% (w/w) chitosan (no Pluronic® F127); and
(vii) TT (5 Lf/ml) and 16.25% (w/w) Pluronic® F127 (no chitosan).

Immunization studies in mice: Balb/c female mice (Harlan), 6 to 8 weeks of age, were used for these studies. Mice were immunized once s.c with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP) labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were collected at weeks 2 and 5, and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data taken two weeks and five weeks following administration is presented in Exhibit C. At five weeks after a single injection, the response in animals receiving TT/F127/chitosan was significantly higher than that to TT in either component alone ($p = 0.02$ vs. TT/chitosan and $p = 0.0006$ vs. TT/F127) with the outlier removed. Figure 13 graphically summarizes IgG antibody titer data for tests on compositions (i), (iv) and (vii).

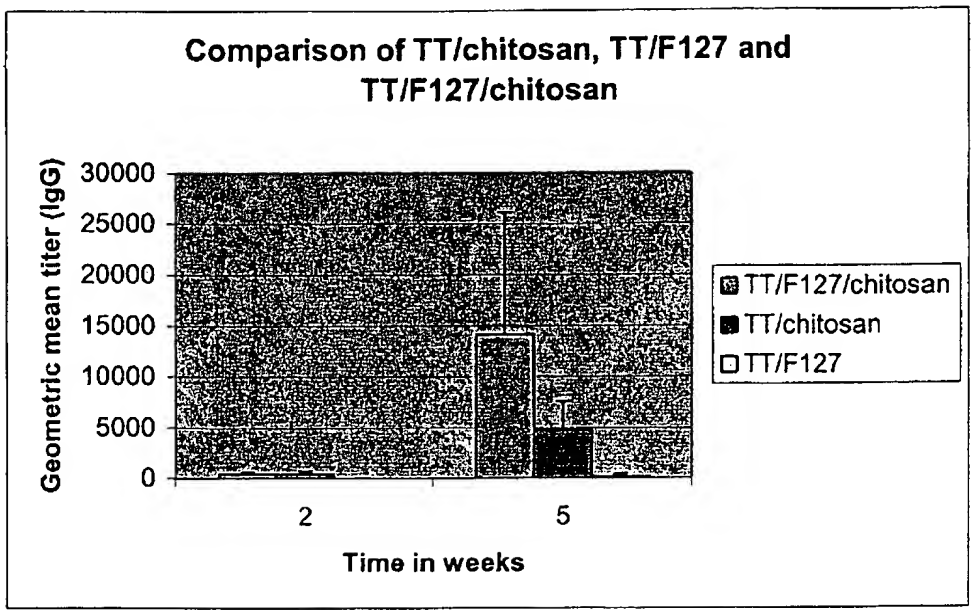


FIGURE 13

EXAMPLE 13: TT with CpG-containing adjuvant in the composition and comparison to other delivery vehicles

Preparation of formulations:. TT and Pluronic® F127 stock solutions were prepared as described in Example 1. ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. Tetanus toxoid (TT; Accurate Chemical & Scientific, Westbury, NY) contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5 Lf/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127);
(iii) TT (5 Lf/ml), 20% (v/w) ImmunEasy™ formulated with glycerol (no Pluronic® F127); and
(iv) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ formulated with incomplete Freund's adjuvant (no Pluronic® F127).

For composition (iii) TT/ImmunEasy™ in glycerol was prepared by mixing glycerol (approximately 99%; Sigma-Aldrich) with premixed TT/ImmunEasy™ in PBS. For composition (iv), TT in incomplete Freund's adjuvant (IFA) was prepared by emulsification of equal volumes of IFA (Sigma-Aldrich) and a 2x TT/ ImmunEasy™ mixture in PBS.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=4) were immunized once s.c. with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over a 28 week period and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following administration is presented in Exhibit D. Figure 14 graphically summarizes the IgG antibody titer data through week 16. Data from a representative experiment indicate that at 4 and 8 weeks, the presence of the Pluronic® F127 polymer significantly enhanced the IgG antibody response to TT compared to antigen/ImmunEasy™ alone ($p = 0.0023$ and 0.029 respectively). Furthermore, the response to TT/F127/ImmunEasy™ was significantly higher than that elicited by TT/ImmunEasy™/IFA ($p = 0.017$ and 0.029 respectively). TT/ImmunEasy™ was also combined with glycerol to make a comparison with another matrix used as both a cryoprotectant and a sustained release vehicle. However, this formulation caused no increase in the anti-TT immune response compared to TT/ImmunEasy™ .

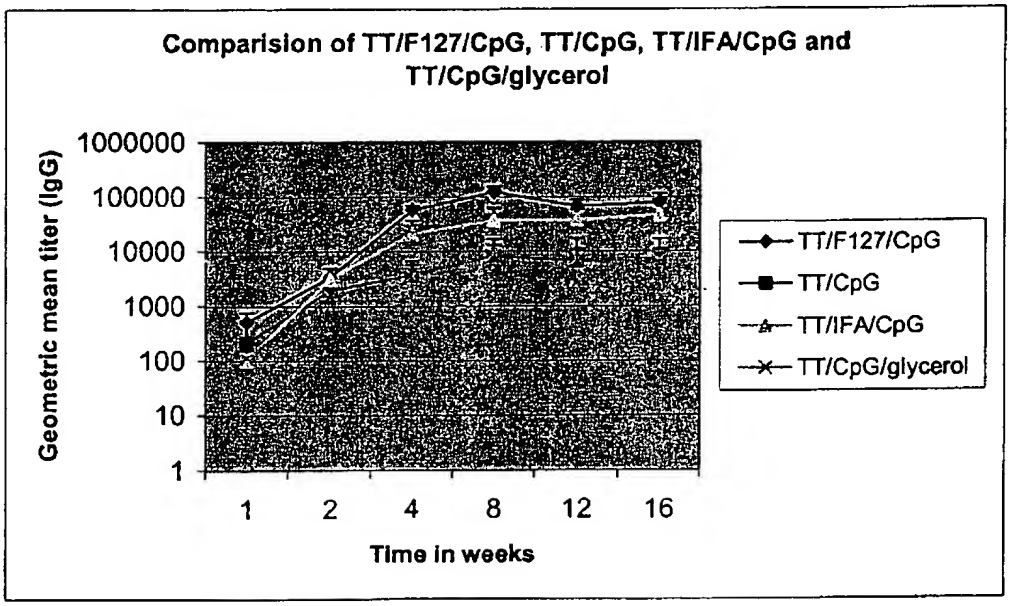


FIGURE 14

EXAMPLE 14: TT with CpG-containing adjuvant in the composition

Preparation of formulations: TT and Pluronic® F127 stock solutions were prepared as described in Example 1. ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20μl, 6.7 μl or 2 μl of the ImmunEasy™ per mouse. Tetanus toxoid (TT; Accurate Chemical & Scientific, Westbury, NY) contained 1058 Lf/ml and 2204 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) TT (5Lf/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(ii) TT (5 Lf/ml), 6.7% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(iii) TT (5 Lf/ml), 2% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127;
(iv) TT (5 Lf/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127);
(v) TT (5 Lf/ml) and 6.7% (v/w) ImmunEasy™ (no Pluronic® F127);
(vi) TT (5 Lf/ml) and 2% (v/w) ImmunEasy™ (no Pluronic® F127; and
(vii) TT (5 Lf/ml) and 16.25% (w/w) Pluronic® F127 (no ImmunEasy™).

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=8) were immunized once s.c. with 0.5 Lf TT in the various formulations on day 0.

Antibody assays: The serum antibody responses to TT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml TT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP) labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were collected at weeks 2, 4, and 8 and assayed for the presence of IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following administration is presented in Exhibit E. Figure 15 graphically summarizes IgG antibody titer data for compositions (iii), (vi) and (vii). The data indicate, for example, that at week 4, the formulation of TT with F127/ImmunEasy™(2%) already elicits a significantly higher response than that elicited by either component mixed with antigen alone ($p = 0.001$ vs. TT/ImmunEasy™ and $p = 0.0003$ vs. TT/F127).

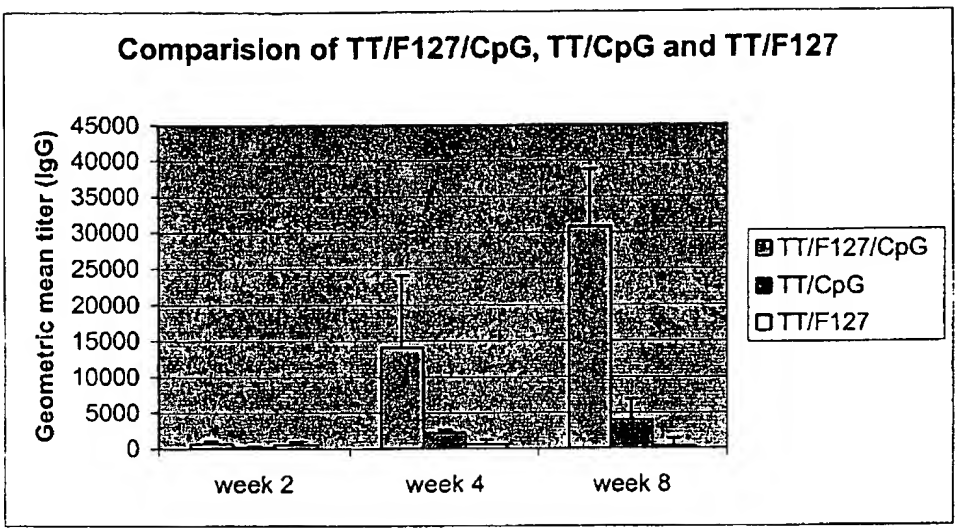


FIGURE 15

EXAMPLE 15: DT with CpG-containing adjuvant in the composition

Preparation of formulations: ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. Diphtheria toxoid (DT; Accurate) contained 2100 Lf/ml and 1667 Lf/mg protein nitrogen. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) DT (1 Lf/dose), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127 and
(ii) DT (1 Lf/dose) and 20% (v/w) ImmunEasy™ (no Pluronic® F127).

Immunization studies in mice: Balb/c female mice (Harlan), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=4) were immunized subcutaneously (s.c) with 1 Lf DT in the various formulations on day 0.

Antibody assays: The serum antibody responses to DT were measured by ELISA. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 10µg/ml DT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over a 32 week period and analyzed for IgG anti-TT antibodies by ELISA. The numerical IgG antibody titer data at various time points following

administration is presented in Exhibit F. Figure 16 graphically summarizes IgG antibody titer data. Data from this experiment indicate, for example, that at 4 and 8 weeks after a single injection, the presence of the Pluronic® F127 polymer and ImmunEasy™ (composition (i)) antigen enhanced the IgG antibody response to DT compared to the use of ImmunEasy™ alone (composition (ii)).

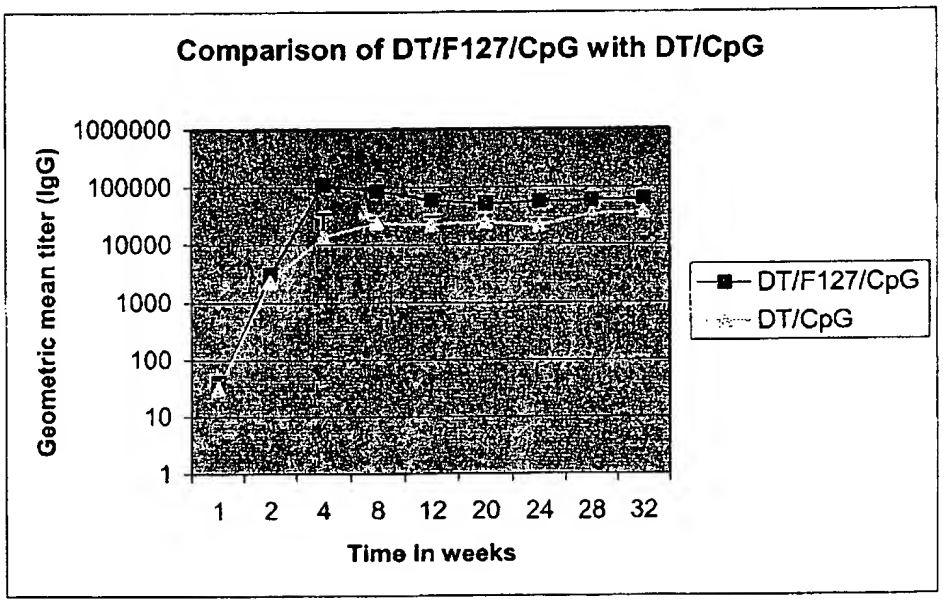


FIGURE 16

EXAMPLE 16: rPA with CpG-containing adjuvant in the composition

Preparation of formulations: ImmunEasy™ containing CpG as an adjuvant was added to formulations in an amount to provide a dose of 20 µl of the ImmunEasy™ per mouse. rPA was obtained from the NIH in the form of a lyophilized protein in 5 mM Hepes, pH 7.4. It was reconstituted in sterile water (USP grade) at 2 mg/ml before formulation. The various stock solutions were mixed together to form vaccine compositions for testing, as follows:

(i) rPA (250µg/ml), 20% (v/w) ImmunEasy™ and 16.25% (w/w) Pluronic® F127 and
(ii) rPA (250µg/ml) and 20% (v/w) ImmunEasy™ (no Pluronic® F127).

Also prepared was a third vaccine composition for testing, as follows:

(iii) rPA adsorbed to aluminum hydroxide (alum) was prepared by adsorption of rPA to Imject® alum (Pierce Endogen, Rockford, IL) according to manufacturer's instructions.

Immunization studies in mice: Balb/c female mice (Harlan, Indianapolis, IN), 6 to 8 weeks of age, were used for these studies. Groups of mice (n=6) were immunized s.c with 25µg rPA in the various formulations on day 0.

Antibody assays: The serum antibody response to rPA was measured by ELISA. The protective capacity of antibodies was measured in vitro using a toxin neutralization assay. For ELISA, wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with 1 μg/ml rPA in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBS/0.1% BSA/0.05% Tween 20 (PBST) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG γ chain specific horseradish peroxidase (HRP)-labeled conjugate (Southern Biotechnology Associates Inc., Birmingham, AL) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (Sigma-Aldrich). Absorbance was read with an EIA reader (Molecular Devices, Sunnyvale, CA). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Serum samples were periodically collected over an 12 week period and analyzed for IgG antibodies by ELISA. Figure 17 graphically summarizes IgG antibody titer data. The data indicate that rPA/F127/ ImmunEasy™ induced an early rise in IgG antibodies and that this response was significantly higher than the response to rPA/alum ($p < 0.05$).

Toxin Neutralization Assay (TNA): Serum samples were tested for their ability to prevent the lethal toxin (protective antigen + lethal factor (LF))-induced mortality of J774A.1 cells (American Type Culture Collection, Manassas, VA). Recombinant LF (rLF) was obtained from the NIH. Aliquots of 0.2 ml cell suspension (6 to $8x10^5$ cells/ml) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) were plated into flat 96-well cell culture plates (Corning Costar, Acton, MA). Serial dilutions of pre- and post-immune serum samples were made in TSTA buffer (50 mM Tris pH 7.6, 142 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20, 2% BSA). PA and LF at final concentrations of 50 and 40 ng/ml respectively were added to each antiserum dilution. After incubation for 1 hour, 10 μl of each of the antiserum-toxin complex mixtures was added to 100 μl of J774A.1 cell suspension. The plates were incubated for 5 hours at 37°C in 5% CO_2. Twenty-five μl of 3-[4,5-dimethyl-thiazol-2-y-]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) at 5 mg/ml in PBS was then added per well. After 2 hour incubation, cells were lysed and the reduced purple formazan solubilized by adding 20% (w/v) sodium dodecyl sulfate (SDS) in 50% dimethylformamide, pH 4.7. OD was read at 570 nm on an EIA reader. The lethal toxin-neutralizing antibody titers of individual serum samples, calculated by linear regression analysis, were expressed as the reciprocal of the antibody dilution preventing 50% of cell death and normalized to a control rabbit anti-rPA hyperimmune serum (NIH). Pre and post-immunization serum toxin neutralization titers were compared by the Sign test. Toxin neutralization titers between groups were compared by the use of the Mann Whitney U test. P values less than or equal to 0.05 were considered to indicate a significant difference.

The functional nature of the immune response to rPA was measured by TNA. The results of these studies (summarized graphically in Figure 18) indicate that formulation of rPA with F127/ ImmunEasy™ induces toxin neutralization titers significantly higher than formulation of rPA with alum ($p=0.002$) and rPA with ImmunEasy™ ($p=0.041$). The TNA titers were measured 8 weeks post immunization.

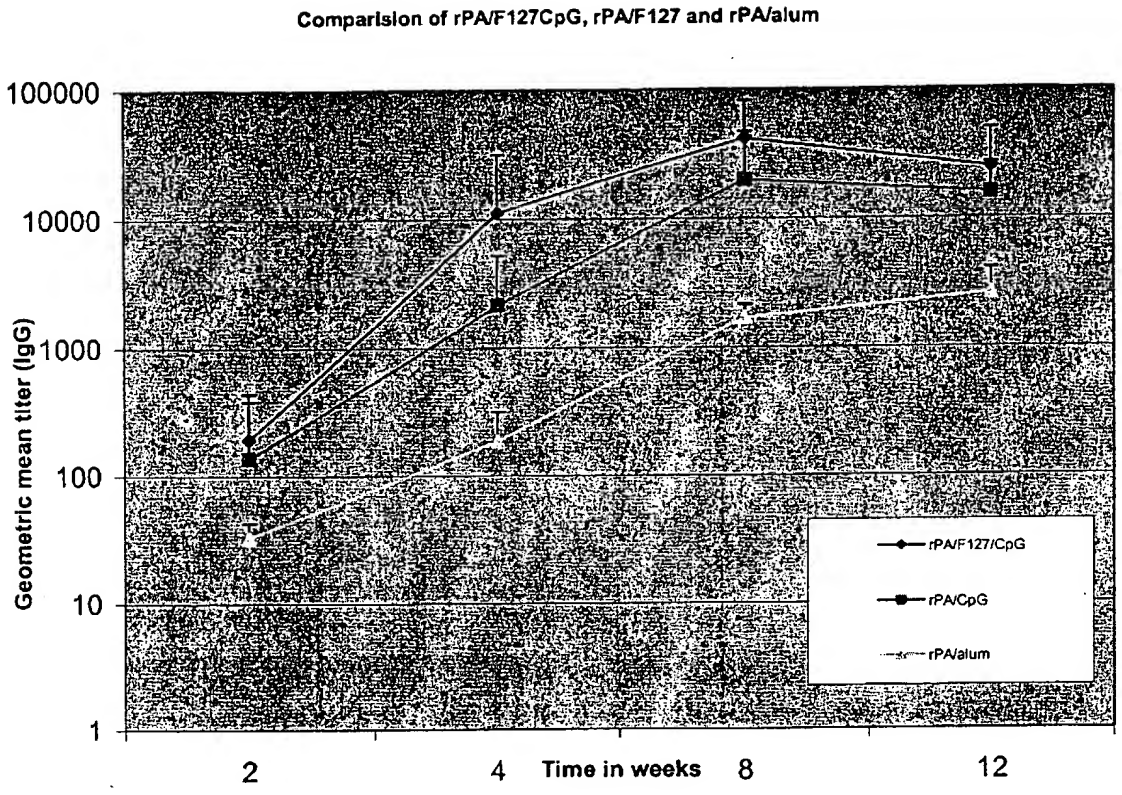


FIGURE 17

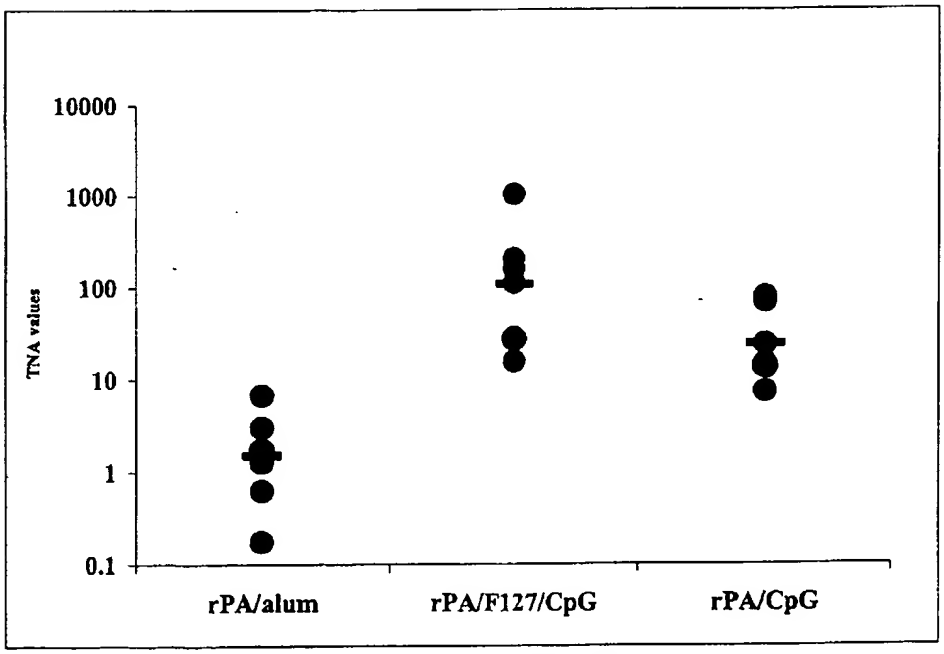


FIGURE 18

EXHIBIT C
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 12 – IgG ANTIBODY TITER DATA – CHITOSAN ADJUVANT

Formulation	**Animal**	**2 week**	**5 week**
0.5Lf TT/F127/Protasan® 0.5%	mouse 1-0	509	13187
	mouse 1-1	741	37839
	mouse 1-2	384	23253
	mouse 1-3	198	5989
	mouse 1-4	356	23257
	mouse 1-5	647	29727
	mouse 1-6	298	15262
	mouse 1-7	429	2170
	Geomean	**413**	**14132**
	Average	**445**	**18836**
	StDev	180	11990
0.5Lf TT/F127/Protasan® 0.17%	mouse 2-0	756	24180
	mouse 2-1	452	17380
	mouse 2-2	481	14339
	mouse 2-3	213	12748
	mouse 2-4	510	8873
	mouse 2-5	493	7991
	mouse 2-6	190	2610
	mouse 2-7	2227	17432
	Geomean	**497**	**11201**
	Average	**665**	**13194**
	StDev	656	6713
0.5Lf TT/F127/Protasan® 0.05%	mouse 3-0	279	2350
	mouse 3-1	184	3623
	mouse 3-2	357	3969
	mouse 3-3	359	6209
	mouse 3-4	92	5001
	mouse 3-5	298	7845
	mouse 3-6	290	23496
	mouse 3-7	310	3949
	Geomean	**252**	**5437**
	Average	**271**	**7055**
	StDev	91	6855

0.5Lf TT/Protasan® 0.5%	mouse 4-0	881	*29039
	mouse 4-1	291	5798
	mouse 4-2	13	4031
	mouse 4-3	698	9871
	mouse 4-4	8	3830
	mouse 4-5	DECEASED	DECEASED
	mouse 4-6	624	7050
	mouse 4-7	256	1840
	Geomean	**162**	**4748**
	Average	**396**	**5403**
	StDev	343	2824
0.5Lf TT/Protasan® 0.17%	mouse 5-0	171	4481
	mouse 5-1	620	8617
	mouse 5-2	409	11043
	mouse 5-3	1382	29896
	mouse 5-4	291	10183
	mouse 5-5	DECEASED	DECEASED
	mouse 5-6	299	DECEASED
	mouse 5-7	95	4431
	Geomean	**337**	**9119**
	Average	**467**	**11442**
	StDev	438	9465
0.5Lf TT/Protasan® 0.05%	mouse 6-0	377	14878
	mouse 6-1	292	9444
	mouse 6-2	179	6055
	mouse 6-3	345	8360
	mouse 6-4	144	3782
	mouse 6-5	176	2365
	mouse 6-6	273	2117
	mouse 6-7	98	2317
	Geomean	**215**	**4862**
	Average	**236**	**6165**
	StDev	100	4517
0.5Lf TT/F127	mouse 7-0	2	2
	mouse 7-1	25	193
	mouse 7-2	68	540
	mouse 7-3	85	252
	mouse 7-4	3	11
	mouse 7-5	163	431
	mouse 7-6	71	407
	mouse 7-7	31	472
	Geomean	**27**	**122**
	Average	**56**	**289**
	StDev	53	207

* outlier by Grubb's test not included in the analysis

EXHIBIT D

TO RULE 132 DECLARATION OF CLAIRE M. COESHOTT

EXAMPLE 13-IgG ANTIBODY TITER DATA-CpG ADJUVANT

Formulation	Animal	1 week	2 week	4 week	8 week	12 week	16 week	20 week	24 week	28 week
0.5Lf TT/F127/ImmunEasy™	mouse 1-0	922	6126	64626	84766	71894	76790	110056	113677	83564
	mouse 1-1	360	2762	71387	131447	42223	40959	114330	119421	127439
	mouse 1-2	345	2562	49756	129251	75097	102047	117018	109813	95376
	mouse 1-3	567	2936	46479	157242	87592	117944	33722	75090	69229
	Geomean	**505**	**3359**	**57152**	**122672**	**66857**	**78439**	**83943**	**102860**	**91572**
	Average	**549**	**3597**	**58062**	**125677**	**69202**	**84435**	**93782**	**104500**	**93902**
	StDev	269	1693	11886	30089	19219	33574	40142	20000	24783
0.5Lf TT/ImmunEasy™	mouse 2-0	50	1230	1762	6365	4520	2646	5554	7029	7165
	mouse 2-1	800	3118	6679	15961	11966	24294	36885	32372	31118
	mouse 2-2	384	1778	3553	5772	3251	4343	18529	32234	33193
	mouse 2-3	126	2233	4337	8919	10607	10262	22403	22312	16906
	Geomean	**210**	**1975**	**3670**	**8504**	**6572**	**7316**	**17077**	**20113**	**18807**
	Average	**340**	**2090**	**4083**	**9254**	**7586**	**10386**	**20843**	**23487**	**22096**
	StDev	338	799	2039	4675	4340	9830	12896	11940	12307
0.5LfTT/Glycerol/ImmunEasy™	mouse 3-0	118	1147	5779	10536	10957	10054	19997	22691	DECEASED
	mouse 3-1	355	1230	934	3138	1003	1745	2145	1432	DECEASED
	mouse 3-2	97	3602	7924	22344	25604	10425	38030	33281	DECEASED
	mouse 3-3	41	879	3993	9092	10536	10754	17639	18127	DECEASED
	Geomean	**114**	**1451**	**3615**	**9053**	**7379**	**6660**	**13024**	**11833**	
	Average	**153**	**1715**	**4658**	**11278**	**12025**	**8245**	**19453**	**18883**	
	StDev	139	1267	2957	8042	10153	4342	14700	13253	
0.5Lf TT/IFA/ImmunEasy™	mouse 4-0	124	3183	24224	34567	45628	46673	100221	104943	73511
	mouse 4-1	138	5819	29907	48098	51500	63457	83854	123394	126116
	mouse 4-2	42	1830	6884	15554	13082	14419	19158	20535	21779
	mouse 4-3	159	2969	43521	74077	64470	112450	179950	214709	220781
	Geomean	**103**	**3167**	**21584**	**37203**	**37520**	**46812**	**73366**	**86926**	**81711**
	Average	**116**	**3450**	**26134**	**43074**	**43670**	**59250**	**95796**	**115895**	**110547**
	StDev	51	1687	15174	24605	21859	40890	66125	79653	84942

EXHIBIT E
TO RULE 132 DECLARATION OF
CLAIRE M. COESHOTT

EXAMPLE 14 – IgG ANTIBODY TITER DATA – CpG ADJUVANT

Formulation	Animal	2 week	4 week	8 week
0.5Lf TT/F127/ImmunEasy™ 20ul	mouse 1-0	2452	4533	6527
	mouse 1-1	8540	10716	21931
	mouse 1-2	6134	7787	16381
	mouse 1-3	10410	5370	18023
	mouse 1-4	7820	18833	178204
	mouse 1-5	5482	DECEASED	DECEASED
	mouse 1-6	13655	25767	191955
	mouse 1-7	7148	155443	111427
	Geomean	**6974**	**14768**	**39903**
	Average	**7705**	**32636**	**77778**
	StDev	3354	54697	81435
0.5Lf TT/F127/ImmunEasy™ 6.7ul	mouse 2-0	1064	43386	227895
	mouse 2-1	3383	100388	101047
	mouse 2-2	1545	129383	173963
	mouse 2-3	2524	107859	146576
	mouse 2-4	1343	13211	658
	mouse 2-5	2197	100320	212735
	mouse 2-6	2930	254421	483347
	mouse 2-7	762	64369	30440
	Geomean	**1761**	**77632**	**76792**
	Average	**1969**	**101667**	**172083**
	StDev	936	72463	149667
0.5Lf TT/F127/ImmunEasy™ 2ul	mouse 3-0	512	19241	42222
	mouse 3-1	201	2002	*273
	mouse 3-2	701	27112	24730
	mouse 3-3	933	27112	23548
	mouse 3-4	708	29535	23802
	mouse 3-5	662	11502	31447
	mouse 3-6	1366	7779	40780
	mouse 3-7	1254	20145	35107
	Geomean	**694**	**14037**	**17065**
	Average	**792**	**18054**	**27739**
	StDev	382	10056	13333

* considered a non-responder removed from plotted data

Formulation	Animal	2 week	4 week	8 week
0.5Lf TT/ImmunEasy™ 20ul	mouse 4-0	3618	2081	2355
	mouse 4-1	2621	1556	2188
	mouse 4-2	5112	7978	12315
	mouse 4-3	10325	20929	131509
	mouse 4-4	5004	5947	1266
	mouse 4-5	9023	7291	19788
	mouse 4-6	4204	15859	658836
	mouse 4-7	6426	4827	136475
	Geomean	**5287**	**6050**	**14429**
	Average	**5792**	**8309**	**46467**
	StDev	2667	6756	57986
0.5Lf TT/ImmunEasy™ 6.7ul	mouse 5-0	523	3678	11148
	mouse 5-1	271	1952	2829
	mouse 5-2	170	2252	6186
	mouse 5-3	368	4555	19027
	mouse 5-4	1082	2005	1479
	mouse 5-5	674	5909	18458
	mouse 5-6	886	3116	17159
	mouse 5-7	458	4309	16667
	Geomean	**476**	**3225**	**8566**
	Average	**554**	**3472**	**11619**
	StDev	311	1411	7247
0.5Lf TT/ImmunEasy™ 2ul	mouse 6-0	254	2217	3005
	mouse 6-1	216	2440	4822
	mouse 6-2	248	1870	2360
	mouse 6-3	253	2122	6307
	mouse 6-4	373	3133	6657
	mouse 6-5	361	1757	2545
	mouse 6-6	456	1978	1897
	mouse 6-7	113	2741	10116
	Geomean	**264**	**2243**	**4034**
	Average	**284**	**2282**	**4714**
	StDev	107	467	2844

0.5Lf TT/F127	mouse 7-0	402	375	553
	mouse 7-1	880	763	965
	mouse 7-2	1025	727	273
	mouse 7-3	100	64	2
	mouse 7-4	1074	1548	1347
	mouse 7-5	1069	851	222
	mouse 7-6	1261	2104	*3364
	mouse 7-7	431	641	682
	Geomean	**623**	**626**	**345**
	Average	**780**	**884**	**926**
	StDev	414	650	1076

* outlier by Grubb's

EXHIBIT F
TO RULE 132 DECLARATION OF
CLAIR M. COESHOTT

EXAMPLE 15 – IgG ANTIBODY TITER DATA – CpG ADJUVANT

Formulation	Animal	1 week	2 week	4 week	8 week	12 week	16 week	20 week	24 week	28 week	32 week
1 Lf DT/ImmunEasy™	mouse 5-0	29	2497	50487	30118	33306	27301	28398	23309	28687	25079
	mouse 5-1	42	3391	6652	22451	24894	25266	26298	9622	32729	49216
	mouse 5-2	58	1325	14953	16828	14477	15250	18644	19928	20736	22238
	mouse 5-3	14	2567	7072	27854	20336	27631	28410	48629	66663	70600
	Geomean	**32**	**2317**	**13728**	**23727**	**22227**	**23219**	**25079**	**21592**	**33753**	**37310**
	Average	**36**	**2445**	**19791**	**24313**	**23253**	**23862**	**25438**	**25372**	**37204**	**41783**
	StDev	19	850	20817	5937	7943	5836	4637	16561	20262	22706
1 Lf DT/F127/ImmunEasy™	mouse 6-0	*9	1540	14847	6070	2928	2470	2880	3532	2250	2161
	mouse 6-1	19	2950	105873	69766	49986	37955	46485	49058	49500	47881
	mouse 6-2	37	2161	129855	98378	64515	54542	55037	57870	57101	59940
	mouse 6-3	44	4051	96437	87278	63688	18668	47777	60742	72827	76451
	Geomean	**23**	**2511**	**66609**	**43668**	**27847**	**17577**	**24358**	**27936**	**26087**	**26241**
	Average	**27**	**2676**	**86753**	**65373**	**45279**	**28409**	**38045**	**42801**	**45420**	**46608**
	StDev	16	1083	49959	41252	29010	22670	23743	26647	30375	31862

*mouse 6-0 omitted from graphic as low responder

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Effectiveness of Natural and Synthetic Complexes of Porin and O Polysaccharide As Vaccines against *Brucella abortus* in Mice

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A single vaccination of mice with a complex of porin and smooth lipopolysaccharide (porin-S-LPS) extracted from virulent *Brucella abortus* 2308 provided significant protection ($P < 0.01$ to $P < 0.001$) against challenge with the same strain, equivalent to that achieved by vaccination with living attenuated *B. abortus* 19. The porin-S-LPS vaccine given without adjuvant or in several adjuvants (trehalose dimycolate and muramyl dipeptide; the pluronic polymer L-121 and muramyl dipeptide; or complexed with Quil A in immunostimulating complexes) provided equivalent protection. In contrast, one vaccination with porin complexed with rough LPS (porin-R-LPS) from a rough mutant of strain 2308 provided no protection with any adjuvant tested. In one experiment, two inoculations with the porin-R-LPS resulted in a low level of protection, probably owing to priming of the animals for production of O-polysaccharide-specific antibodies. However, one vaccination with rough-strain porin covalently bound to purified O polysaccharide conferred protection equal to that obtained with natural complexes of porin-S-LPS or with living strain 19. A synthetic vaccine containing long chains of O polysaccharide was more effective than one prepared with short chains. Protective vaccines caused the formation of increased concentrations of circulating O-polysaccharide-specific antibodies, although there were individual exceptions to the quantitative association between O-polysaccharide-specific antibodies and protection. Antibodies specific for porin or R-LPS were found in negligible quantities in vaccinated mice. These results provide additional evidence that the O polysaccharide will constitute an essential component of an effective subcellular vaccine against *B. abortus* and that O-polysaccharide-specific antibodies play an important role in protective immunity in brucellosis.

Disease caused by *Brucella abortus* affects humans and a number of animal species, particularly cattle (43). Attenuated *B. abortus* 19 has been used for many years to vaccinate cattle against brucellosis. Among the major drawbacks of this vaccine are its ability to cause disease in cattle (14) and humans (56) and to induce O-polysaccharide (OPS)-specific antibodies (O antibodies) which interfere with the interpretation of serodiagnostic tests (43).

A principal goal of research in this laboratory (31, 53–55) and others (5, 6, 13, 15, 47, 48) is the development of a subcellular vaccine for bovine brucellosis. Such a vaccine would not be commercially acceptable unless it provided an advantage over strain 19 such that the antibody response which it induced could be clearly distinguished from that evoked by virulent field strains. This could be accomplished easily if it was possible to design an effective vaccine which excluded the OPS. However, recent studies with the murine model system have provided evidence by active (31) and passive (26, 31, 32) immunizations that O antibodies play an important role in protection.

It is not yet certain, however, that the OPS of *B. abortus* represents an indispensable element of an effective vaccine. This question must be resolved to provide direction for future efforts on development of subcellular vaccines. The experiments reported here were done in pursuit of that objective. We performed active immunizations with simple and defined vaccines with or without the OPS. The design of the experiments was based on a hypothesis proposed by one of us (52) that the simplest vaccine which could provide protection equivalent to strain 19 would have to include the OPS and a protein such as porin to which smooth lipopolysaccharide (S-LPS) was bound in the living bacterium. The protein would at once serve as a carrier for the induction of O antibodies and, in the presence of appropriate adjuvants, induce cell-mediated immune responses required for protection (52).

(The data in this report are taken in part from the Ph.D. thesis of M. J. Eis, Cornell University, Ithaca, 1988.)

MATERIALS AND METHODS

Mice. Five-week-old female BALB/cByJ mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and held for 1 week before use.

Bacterial strains for vaccination and challenge. *B. abortus* smooth strain 2308 of known virulence was used for challenge infections. In some experiments, vaccination or challenge was performed with vaccine strain 19 (Biologics Division, U.S. Department of Agriculture). Stock cultures were prepared and stored as described previously (31), and inocula were prepared from a freshly thawed vial which was diluted in a predetermined manner in sterile phosphate-buffered saline (PBS) to yield an infecting dose of 5×10^4 bacteria per 0.1 ml.

Rough stains of 2308 were provided by G. G. Schurig (Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Va.) and B. L. Deyoe (National Animal Disease Center, Ames, Iowa). These are referred to as strains RB51 and NADC, respectively.

Antigens for vaccines and immunoassays. (i) Porins. Native porin proteins were extracted and purified from smooth

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strain 2308 and rough strain NADC by a modification of previously described methods (51). Porins were freed of detergents, lyophilized, and stored in a desiccator at room temperature (55). Porin from rough strain RB51, which was used for production of synthetic vaccines, was produced by a modification of the methods of Verstreate et al. (51) in which bacterial cells were disrupted by vortexing with glass beads for 5 min at 0°C (Bead-Beater; Biospec Products, Bartlesville, Okla.) and proteins separated by ion-exchange chromatography were subjected to chromatofocusing (PBE 94; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) prior to gel filtration. Denaturation of the porin was required for successful coupling to the OPS. This was accomplished by heating the protein at 100°C for 15 min in 10 mM Tris buffer (pH 8.0) containing 0.1 M NaCl, 0.1% Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.), 0.02% NaN_3, and 2% sodium dodecyl sulfate. Sodium dodecyl sulfate and NaN_3 were removed by gel filtration on Sephacryl S-300 (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl and 0.015% Zwittergent 3-14. Porin-containing fractions were pooled and concentrated by ultrafiltration during which the buffer was replaced with carbonate buffer (0.1 M, pH 9.2) used in the coupling reaction.

(ii) **S-LPS.** S-LPS (fraction f6) was produced from smooth strain 2308 by extraction in hot phenol followed by treatment with guanidinium thiocyanate (38).

(iii) **OPS.** LPS was extracted from cells of *B. abortus* smooth strain 2308 by the method of Moreno et al. (35). A solution of purified LPS in 2% acetic acid was refluxed at 100°C (8 h), cooled to 4°C, and diluted with an equal volume of 70% trichloroacetic acid to precipitate proteins. After centrifugation (17,000 × *g*, 15 min), the supernatant was combined with methanol (5 volumes) at 4°C, stirred (16 h), and centrifuged (17,000 × *g*, 20 min). The resulting pellet was dissolved in distilled deionized water (DDW) (100 ml), dialyzed against DDW for 2 days, and lyophilized. The product was suspended in 20 mM Tris chloride buffer (pH 8.0, 100 ml) and centrifuged (17,000 × *g*, 10 min), and the supernatant was applied to a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Eluted fractions (absorbing at 210 nm) were combined, and OPS was precipitated by the addition of 5 volumes of methanol at 4°C. After centrifugation (17,000 × *g*, 15 min), the pellet was dissolved in DDW, dialyzed against DDW, and lyophilized, affording pure OPS whose ^{13}C and ^{1}H nuclear magnetic resonance and infrared spectra were in agreement with published values (10).

(iv) **Preparation of long and short OPS oligomers.** Anhydrous HF (15 ml) was added to a 2-oz (59-ml) polyethylene bottle with a Teflon stir bar under argon charged with 775 mg of purified OPS. The bottle was tightly capped, and the colorless solution was stirred at −20°C for 2.5 h. The HF was removed under anhydrous conditions with a stream of argon and then under vacuum (0.1 torr [13.33 Pa], minimum 1 h) until the reddish solid became colorless. This residue was dissolved in DDW (10 ml) and submitted to gel filtration on a column (2.6 by 95 cm) of Bio-Gel P-4 (200/400 mesh) at a flow rate of 10 ml/h and with UV detection at 210 nm. Fractions were collected at ca. 190 to 200 ml (void volume), ca. 200 to 300 ml, and ca. 300 to 400 ml to afford, after lyophilization, unreacted OPS (42 mg, 6%), long α-1-fluoro-OPS oligomers (306 mg, 41%), and short α-1-fluoro-OPS oligomers (214 mg, 28%), respectively. Short-chain oligomers were calculated to contain between 5 and 20 sugar residues, while the size of long-chain oligomers varied from about 20 sugar residues to native length.

Long (382 mg) and short (219 mg) α-1-fluoro-OPS oligomers were suspended in 2.6 and 1.4 ml, respectively, of freshly distilled 2-azidoethanol. (It must be emphasized that 2-azidoethanol is explosive. Preparations of this material should never be heated above 60°C, and all distillations must be performed behind a safety shield.) Additional 2-azidoethanol (2.6 and 1.4 ml, respectively) saturated with anhydrous HCl was added to each vessel. After being capped tightly, the solutions were stirred at 25°C for 12 h. Hydrogen chloride was removed in vacuo at 20°C and residual 2-azidoethanol was removed under vacuum (0.1 torr) at 25°C over ca. 24 h. Each crude product was dissolved in ca. 5 ml of DDW and applied to a Bio-Gel P-4 column (2.6 × 95 cm) (200/400 mesh) with a flow rate of ca. 8 ml/h. Lyophilization afforded long azidoethyl-OPS (300 mg, 79%) and short azidoethyl-OPS (200 mg, 91%).

Catalytic hydrogenation of the long (317 mg) and short (324 mg) azidoethyl-OPS was done in separate 100-ml round-bottom flasks by adding 10% Pd on C (150 mg) to solutions of each sample in ethanol-water (75 ml:15 ml). The mixtures were stirred while H_2 was bubbled through E-fritted tubes for 36 to 48 h. Ethanol was removed in vacuo and was replaced by 100 ml DDW. Catalyst was removed by filtration through a 4-mm pad of Celite, and the solutions were lyophilized to afford both long 2-aminoethyl-OPS (286 mg, 90%) and short 2-aminoethyl-OPS (260 mg, 80%).

A 100-mg sample of either long or short 2-aminoethyl-OPS in DDW (2.5 ml) was added to a solution of di-2-hydroxypyridyl thionocarbonate (23) (100 mg, 0.43 mmol) in DMF (5 ml) at 15°C. The solution was stirred at 25°C (1 h) and then diluted with 100 ml of DDW, washed with CH_2Cl_2 (20 ml, five times) and lyophilized. The residue was dissolved in DDW (5 ml) and centrifuged and the supernatant was passed through a column (2.6 by 100 cm) of Bio-Gel P-4 (200/400 mesh) at 8 ml/h, with UV detection at 248 nm. Lyophilization afforded long α-1-O(2-isothiocyanatoethyl)-OPS (NCS-OPS) (86 mg, 86%) or short NCS-OPS (75 mg, 75%).

Satisfactory infrared spectra were obtained from the compounds at each stage of the reaction.

(v) **Long and short porin-OPS glycoprotein conjugates.** Long NCS-OPS (50 mg) in 0.230 ml of 0.1 M carbonate buffer (pH 9.2) was added to a 2-ml polyethylene centrifuge tube containing 5 mg of denatured porin in 0.125 ml of the above buffer. The tube was capped, and the solution was gently vortexed for 15 s. After standing at room temperature for 36 h, the solution was applied directly to the top of a gel filtration column (2.6 by 96 cm) of Sephacryl S-300 equilibrated with 50 mM phosphate buffer (pH 7.5)–0.1 M NaCl–0.015% Zwittergent 3-14. The flow rate was adjusted to 18 ml/h with collected fractions of 6 ml and UV detection at 280 nm. Pooled fractions (120 ml) containing glycoprotein were concentrated by ultrafiltration on a YM-10 membrane, followed by buffer exchange with 0.005% Zwittergent in DDW to produce a final volume of 4.75 ml (0.66 mg of protein per ml by Lowry assay) which was made 0.02% in NaN_3 and stored at 4°C. Short-chain porin-OPS conjugate (4 ml [0.41 mg/ml]) was produced by identical methods from a mixture of 35 mg of short NCS-OPS and 5 mg of denatured porin. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses of long- and short-chain conjugates demonstrated little or no native porin. Whereas porin produced two closely spaced, well-resolved bands, the conjugates produced an unresolved smear of higher-molecular-weight materials which terminated just above the principal porin band (M. J. Eis, Ph.D. thesis).

(vi) **Conversion of α-1-O-(2-aminoethyl)-OPS oligomers to**

α-1-O-(*N*-stearoyl-2-aminoethyl)-OPS glycolipid. Stearoyl chloride (86 mg, 0.28 mmol) in DMF (3 ml) was added dropwise to a solution of long 2-aminoethyl-OPS (125 mg) in DMF (6 ml) and pyridine (4.5 ml). The reaction mixture was stirred at 25°C for 6 h and then diluted with methanol (5 ml) and stirred for an additional 30 min. The bulk of solvent was removed in vacuo at 45°C. The residue was dissolved in DDW (200 ml), and the solution was adjusted to pH 8.0 with 1 M NaOH and lyophilized. The resulting waxy white solid was collected on an E-fritted glass filter and washed with CH_2Cl_2 (100 ml twice). Rinsing of the frit with DDW (100 ml once) and lyophilization of the resulting soapy solution afforded 119 mg of crude N,O-stearoylated glycolipid. To hydrolyze O stearoyl groups, 100 mg of this material was redissolved in 10 ml of 0.1 M NaOH and stirred at 55°C for 3.5 h. The solution was cooled, diluted to 100 ml with DDW, adjusted to pH 5.0 with 5% HCl, and lyophilized to yield a waxy solid. This was washed with CH_2Cl_2 (100 ml twice) atop an E-fritted glass filter and dissolved in DDW (20 ml), and the resulting solution was dialyzed in 3,200-molecular-weight-cutoff dialysis tubing against two 1,000-ml volumes of DDW over 2 days. Lyophilization of the dialyzed solution afforded 40 mg of glycolipid which produced satisfactory nuclear magnetic resonance and infrared spectra.

Adjuvants. (i) TDM and MDP. Trehalose dimycolate (TDM) and *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (bMDP) were preparations used previously (31). Preparation of vaccines with these adjuvants was as described previously (31) except that squalane (Sigma Chemical Co., St. Louis, Mo.) was used in place of mineral oil.

(ii) ISCOMs. Immunostimulating complexes (ISCOMs) were prepared by published methods by Morein and colleagues (34, 45) with porins of the smooth and the NADC rough strain of *B. abortus* 2308.

(iii) L-121 and MDP. The method of vaccine preparation for L-121 and MDP was kept as similar as possible to that used for TDM and MDP. bMDP solubilized in methanol was added to dried antigen in a glass homogenizer. After the methanol was completely evaporated, the pluronic polyol L-121 (20; 21) (BASF Wyandotte, Co., Parsippany, N.J.) and squalane were added and homogenized as previously described (55). A solution of PBS with 0.2% Tween 80 (PBS-Tween) was then added and emulsified with the oil mixture (55). Each mouse received 5 μg of antigen, 10 μg of bMDP, 2.5 μg of L-121, 5 μl of squalane, and 95 μl of PBS-Tween.

Blood sampling. Blood samples were taken retro-orbitally and in most experiments were pooled within treatment groups. Sera were stored at −20°C. Thawed samples were diluted 1:25 in PBS and passed through a bacterial filter before use.

Experimental design. Unless otherwise stated, 6-week-old mice were vaccinated subcutaneously (s.c.) 4 weeks prior to intravenous (i.v.) challenge with approximately 5×10^4 live *B. abortus* cells. Blood samples were taken on one or more occasions during the course of some experiments. Quantitative cultures for viable *B. abortus* were performed on spleens at 1 and 4 weeks postinfection (p.i.) by the methods of Montaraz and Winter (31). Spleens from mice vaccinated with live strain 19 before challenge with strain 2308 were cultured on plates containing 0.1% erythritol to inhibit the growth of strain 19 (31).

Monoclonal antibody. A monoclonal antibody specific for the A epitope of the *B. abortus* OPS (8, 10) was provided as ascites fluid by James Douglas (University of Hawaii, Honolulu). The titer of this antibody with the f6 fraction in a standard plate enzyme-linked immunosorbent assay (ELISA) was 25,160.

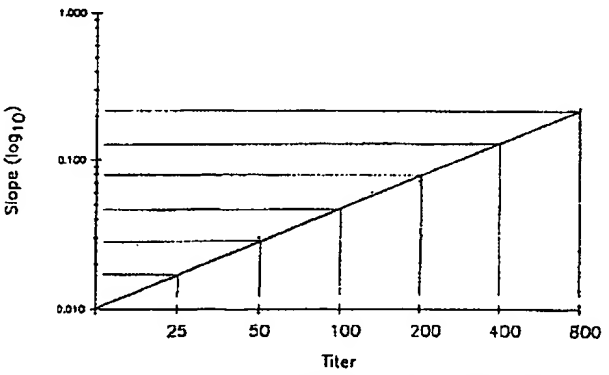

FIG. 1. Linear relationship between ELISA antibody titers and log of slope values.

Enzyme immunoassays. Antibodies were measured by an indirect kinetics-based ELISA (55). Plates were incubated overnight at 4°C with 500 ng of antigen per well of carbonate buffer (pH 9.6). After four washes with PBS containing 0.05% Tween 20, each well received 100 μl of serum diluted 1:25 in PBS. Plates were incubated for 60 min at 37°C and then washed four more times. A 100-μl portion of Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy and light chains) (Organon Teknika, West Chester, Pa.) diluted 1:1,000 in PBS-Tween 20 was added to each well followed by incubation for 30 min at room temperature. After final washing, 100 μl of 2,2'-azino-di-(3-ethylbenzthiazolinesulfonic acid) and hydrogen peroxide substrate solution was added. The slope of colored product development was measured in the linear response phase of enzyme-substrate interaction and was determined by computer-timed absorbance readings at 410 nm 2, 4, and 6 min after substrate addition. Standard plate ELISA was conducted by the same procedure except that plates were incubated for 30 min at 37°C with sera and 20 min at 37°C after conjugate addition. Colored product development was terminated after 20 min at room temperature by the addition of 100 μl of 0.1 M HF per well. Absorbance was read at 410 nm on a Minireader II (Dynatech Corp., Chantilly, Va.). Antibody titer was considered to be the highest dilution at which absorbance readings were 0.2 units greater than the control.

The kinetics-based ELISA was used because it enables more precise comparisons to be made among samples (22). For ease of understanding, however, the approximate conversions of the data from slope values to titers are included in the tables and will be used in referring to antibody responses. To obtain these conversions, we prepared samples from a positive control serum which gave titers of 50, 200, and 800, respectively, in the standard plate ELISA. Slope values were then derived from 1:25 dilutions of each of the three samples, because 1:25 was standard dilution used for antibody determinations by kinetics-based ELISA. A nomograph established by regression analysis demonstrated a linear relationship between the log of slope values and antibody titers ($r^2 = 1.0$) (Fig. 1).

A competitive assay was developed to determine the comparative amounts of antibody-reactive OPS on the antigens used for vaccination. Antigen-coated microtiter wells were prepared by adding 100 μl of f6 (2.5 μg/ml in carbonate buffer [pH 9.6]) per well and freezing the plate at −20°C. Plates were thawed 1 to 2 h before use. Well-washing

TABLE 1. Estimation of S-LPS and OPS content of vaccines by a competitive inhibition test

Vaccine	% S-LPS[a]	% OPS[a]
Porin-S-LPS[b]	15.0	10.7
Porin-R-LPS (NADC)[c]	0.01	0.007
Porin-R-LPS (RB51)[c]	0.01	0.007
R-porin-OPS (long)[d]		58.2
R-porin-OPS (short)[d]		10.7

[a] S-LPS fraction of f6 was used as a standard. Calculations were based on the content of S-LPS and OPS in the f6 sample as 94% (35, 38) and 67% (35), respectively.
[b] Native porin from smooth strain 2308.
[c] Native porin from rough 2308 strains NADC and RB51.
[d] Short- or long-chain OPS covalently linked to denatured porin from strain RB51.

procedures were as described above. Duplicate external dilutions of 100-μl samples of test antigen were incubated with 100 μl of a 1:800 dilution of monoclonal antibody for 3 h prior to the addition of 100 μl of the mixture to a washed antigen-coated well for 1 h. After washing, 100 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Organon Teknika) was added for a 3-h incubation. After washing, 100 μl of *p*-nitrophenyl disodium phosphate was added at 1 mg/ml in diethanolamine buffer (pH 9.8), and slope values were determined from readings at 405 nm 2, 4, and 6 min later. A standard curve was prepared with samples of f6 in twofold dilutions from 20 to 0.31 μg. Test samples were assayed at concentrations of 40, 20, and 10 μg. An equation for the standard curve was determined by linear regression and then used to calculate the concentration of OPS in the test samples. An r^2 of 0.97 was found for the regression after log transformation of the antigen concentrations. The f6 standard was assumed to contain 94% S-LPS (35, 38) and 67% OPS (35).

Statistical methods. A log value of bacteria in each spleen was obtained by averaging the triplicate counts following log conversion (31). Statistical significance was determined by Fisher's protected least-significant-difference test (42). Correlations were analyzed by Pearson's product moment test (42).

RESULTS

OPS content of antigens. Porin extracted from smooth strain 2308 contained an estimated 15% associated S-LPS (Table 1), consistent with prior data (51). The two preparations of porin derived from rough strains of 2308 contained trace quantities of S-LPS according to the competitive inhibition assay (Table 1). However, this interpretation must be qualified because these values lie within the range of experimental error of the test. The synthetic vaccines prepared by binding long or short OPS chains to porin of rough strain RB51 were composed of approximately 58.2 and 10.7% OPS, respectively (Table 1). Both of these preparations when used as antigens in ELISAs produced reactions with monoclonal O antibodies equivalent in titer to that obtained with the O glycolipid (data not shown).

Vaccination trials with natural complexes of porin and LPS. Vaccination with 30 μg of porin-S-LPS conferred significant protection against challenge infections with either *B. abortus* 2308 or 19 at both 1 and 4 weeks p.i. (Table 2). The magnitude of protection was not significantly enhanced at either time period by the inclusion of TDM and MDP adjuvant in the vaccine. In contrast, vaccination with the same quantity of porin-R-LPS with or without adjuvant provided no significant protection against either challenge strain (Table 2).

Vaccination trials with porins incorporated into ISCOMs produced the same results. Porin-S-LPS ISCOMs provided significant protection against strain 2308 at 1 and 4 weeks p.i. even at the lowest dose tested (40 ng) (Table 3). At 1 week p.i., protection provided by 30 μg of porin-S-LPS in PBS was not different from that produced by 5 μg of ISCOMs, but was significantly greater ($P < 0.001$ to $P < 0.01$) than protection provided by all lower doses. Decreasing doses of ISCOMs produced successively lower levels of protection (Table 3) which were significantly different from each other ($P < 0.001$ to $P < 0.05$) except for the 1-μg and 200-ng doses. At 4 weeks p.i., no significant differences in protection could be ascribed to dose of vaccine or presence of adjuvant. Again, porin-R-LPS in ISCOMs produced no significant protection at any dose used (Table 3).

An experiment was next performed to test the effect of different adjuvants in enhancing protection provided by a uniform dose of porin-S-LPS. A 5-μg dose of porin-S-LPS conferred significant protection against strain 2308 at 1 and 4 weeks p.i. whether given as ISCOMs, with TDM and MDP, with L-121 and MDP, or without adjuvant (Table 4). The incorporation of adjuvants did not increase protection except at 1 week p.i. when the vaccine with TDM and MDP

TABLE 2. Vaccination with porin from smooth or rough strains of *B. abortus* 2308 with or without adjuvant[a]

Expt	Vaccine (μg)[b]	Adjuvant	Challenge strain	Log_{10} brucellae in spleens (mean ± SD)	
				1 wk p.i.	4 wk p.i.
1	None	None	2308	6.57 ± 0.94	7.01 ± 0.64
	Porin-S-LPS (30)	None	2308	4.18 ± 0.82***	5.77 ± 1.25*
	Porin-S-LPS (30)	TDM-MDP	2308	3.88 ± 0.81***	5.34 ± 1.08**
	Porin-R-LPS (30)	None	2308	7.20 ± 0.15 (NS)	7.11 ± 0.25 (NS)
	Porin-R-LPS (30)	TDM-MDP	2308	6.00 ± 1.33 (NS)	6.97 ± 0.28 (NS)
	None	TDM-MDP	2308	7.14 ± 0.10 (NS)	6.80 ± 0.32 (NS)
2	None	None	19	6.83 ± 0.45	6.37 ± 0.29
	Porin-S-LPS (30)	None	19	3.16 ± 0.68***	3.69 ± 1.45***
	Porin-S-LPS (30)	TDM-MDP	19	3.43 ± 1.45***	3.72 ± 1.68***
	Porin-R-LPS (30)	None	19	7.20 ± 0.16 (NS)	6.25 ± 0.27 (NS)
	Porin-R-LPS (30)	TDM-MDP	19	5.93 ± 1.60 (NS)	5.60 ± 1.36 (NS)
	None	TDM-MDP	19	6.95 ± 0.27 (NS)	5.79 ± 0.66 (NS)

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308 or strain 19. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, not significant.
[b] Porin from smooth strain (S) or from rough strain NADC (R) 2308.

TABLE 3. Vaccination with porin from smooth or rough strains of *B. abortus* 2308 in ISCOMs[a]

Expt	Vaccine (μg)[b]	Adjuvant	Log_{10} brucellae in spleens (mean ± SD)	
			1 wk p.i.	4 wk p.i.
1	None	None	7.14 ± 0.12	6.99 ± 0.37
	Porin-S-LPS (30)	None	3.81 ± 0.10***	5.57 ± 0.96*
	Porin-S-LPS (5)	ISCOM	3.39 ± 0.22***	4.38 ± 0.85***
	Porin-S-LPS (1)	ISCOM	4.59 ± 0.54***	5.41 ± 0.84**
	Porin-S-LPS (0.2)	ISCOM	4.86 ± 0.39***	4.61 ± 0.85***
	Porin-S-LPS (0.04)	ISCOM	5.40 ± 0.56***	5.20 ± 1.11**
2	None	None	7.17 ± 0.16	7.04 ± 0.48
	Porin-R-LPS (30)	None	7.17 ± 0.13 (NS)	6.97 ± 0.53 (NS)
	Porin-R-LPS (5)	ISCOM	7.41 ± 0.08 (NS)	7.42 ± 0.14 (NS)
	Porin-R-LPS (1)	ISCOM	7.09 ± 0.75 (NS)	6.98 ± 0.31 (NS)
	Porin-R-LPS (0.2)	ISCOM	7.03 ± 0.40 (NS)	6.56 ± 1.04 (NS)
	Porin-R-LPS (0.04)	ISCOM	7.23 ± 0.28 (NS)	6.99 ± 0.59 (NS)

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.
[b] Porin from smooth strain 2308 (S) or rough strain NADC (R) 2308.

provided greater protection than antigen in PBS ($P < 0.05$). The efficacy of vaccines containing different adjuvants did not differ significantly among each other.

Vaccination trials with synthetic complexes of porin and OPS. The results obtained with natural complexes of porin and LPS supported the hypothesis that the OPS was an essential component of an effective vaccine. We therefore proceeded to determine whether porin-R-LPS, an ineffective vaccine, could be converted into an effective one by the addition of OPS.

In the first experiment, mice were vaccinated with 30 μg of synthetic complexes of R-porin coupled with long (R-porin-OPS-long) or short (R-porin-OPS-short) chains of OPS in the TDM and MDP adjuvant. The synthetic complexes conferred significant protection against challenge infection with strain 2308 at 1 week p.i. ($P < 0.001$) (Table 5). Protection was comparable to that obtained in mice vaccinated with porin-S-LPS (Table 5) and was significantly greater than that due to adjuvant alone (Table 5) ($P < 0.001$). Mice vaccinated with porin-R-LPS were not protected (Table 5). In another trial, protection was demonstrated at 1 week ($P < 0.001$) and 4 weeks ($P < 0.05$) in mice vaccinated with 30 μg of R-porin-OPS-long without adjuvant, whereas no protection was afforded to mice vaccinated with the same lot of denatured porin which had not been conjugated to OPS (Table 6).

Despite initial success achieved with the R-porin-OPS short-chain vaccine (Table 5), two attempts to reproduce protection with this preparation failed (data not shown). An experiment was therefore performed to determine whether revaccination would improve the effectiveness of this vaccine. At the same time, a direct comparison was made between the natural and synthetic porin vaccines and living strain 19. Mice vaccinated twice with porin-S-LPS or R-porin complexed with either long- or short-chain OPS were protected following challenge with strain 2308 ($P < 0.01$ to $P < 0.001$) (Table 7). Levels of protection obtained with the subcellular vaccines were in no instance significantly different from that achieved with strain 19. In this experiment, low (0.74 logs) but significant ($P < 0.05$) protection was also achieved with the porin-R-LPS vaccine (Table 7). Protection achieved with the porin-R-LPS vaccine was, however, significantly below that obtained with strain 19, porin-S-LPS, or R-porin-OPS-long vaccines ($P < 0.01$).

Association of protection with induction of O antibodies. Pooled sera taken just prior to challenge infection from mice vaccinated 4 weeks earlier with 5 μg of porin-S-LPS in PBS contained O antibodies at a titer of 100, and incorporation of adjuvants increased the titers four- to eightfold (Table 4). All these vaccines conferred high levels of protection, as noted above (Table 4).

In the experiment reported in Table 7, pooled sera were collected from each group prior to the first and second vaccinations and prior to challenge infection. Individual samples were taken prior to killing the mice at 1 week p.i. A single vaccination with porin-S-LPS resulted in a titer of O antibodies of 200 4 weeks later (Table 7). This was increased to 800 as a result of revaccination and remained at this level

TABLE 4. Vaccination with porin from smooth strain 2308 in different adjuvants[a]

Vaccine (μg)[b]	Adjuvant	Antibody level[c]		Log_{10} brucellae in spleens (mean ± SD)	
		Slope (10^3)	Titer	1 wk p.i.	4 wk p.i.
None	None			7.20 ± 0.15	7.17 ± 0.48
Porin-S-LPS (5)	None	62	100	3.90 ± 0.45***	5.55 ± 0.66**
Porin-S-LPS (5)	ISCOM	241	800	3.46 ± 0.83***	5.29 ± 1.07**
Porin-S-LPS (5)	TDM-MDP	242	800	3.25 ± 0.39***	4.71 ± 0.83***
Porin-S-LPS (5)	L-121-MDP	134	400	3.47 ± 0.20***	4.25 ± 0.68***

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$.
[b] Porin from smooth strain 2308.
[c] Mice were bled retro-orbitally prior to challenge. Pooled serum samples were tested in kinetics-based ELISA against fraction f6. Numbers reported are slopes, and titers were estimated from slopes (Fig. 1).

TABLE 5. Vaccination with naturally occurring porins or synthetic porin-OPS complexes[a]

Vaccine (μg)	Adjuvant	Log_{10} brucellae in spleens (mean ± SD) (1 wk p.i.)
None	None	6.86 ± 0.08
Porin-S-LPS[b] (30)	TDM-MDP	4.01 ± 1.07***
Porin-R-LPS[b] (30)	TDM-MDP	6.81 ± 0.38 (NS)
R-porin-OPS (short)[c] (30)	TDM-MDP	3.57 ± 0.23***
R-porin-OPS (long)[c] (30)	TDM-MDP	3.22 ± 0.31***
None	TDM-MDP	5.34 ± 0.84**

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$; NS, not significant.

[b] Porin from smooth (S) or rough strain NADC (R) 2308.

[c] Short- or long-chain OPS covalently linked to porin from rough strain RB51.

after challenge infection (Table 7). Although vaccination with porin-R-LPS produced only the most marginal increase after the second inoculation, it is notable that the titer in this group following challenge was fourfold greater than that in the nonvaccinated group (Table 7). In contrast with the high antibody response (titer of 400) observed after the first vaccination with the synthetic long-chain vaccine, a single vaccination with the short-chain vaccine produced no increase in O antibodies (Table 7). A second inoculation of the short-chain vaccine caused the appearance of circulating O antibodies (titer of 100), and the titer of antibodies in this group following challenge was fourfold greater than that in the nonvaccinated group (Table 7). Antibody levels (measured as slope values) at 42 days were correlated in an inverse fashion with mean log numbers of bacteria cultured from the spleens of each treatment group ($r^2 = -0.828$; $P < 0.05$).

Marginal increases in antibodies specific for the porin-R-LPS complex were detected in groups immunized with subcellular vaccines (Table 7). In only one instance did these attain a titer of 25 (Table 7).

Further analyses were performed on individual sera of four of the groups to determine whether levels of O antibodies at 1 week p.i. were correlated with protection in individual animals. For this purpose, the O glycolipid was used as the antigen, primarily to ensure that antibody responses produced by porin-R-LPS were not directed against porin or lipid A determinants. Analyses of the 35-day and 42-day serum pools with f6 and O glycolipid antigens demonstrated comparable reactions (Table 8) which were highly correlated ($r^2 = 0.968$; $P < 0.01$). Slopes of reactions against O glycolipid were on the average 12.5% lower than those with f6. Levels of O antibodies were very similar in mice numbers 13 to 18, immunized with the long-chain synthetic vaccine, in which spleen counts showed little variation (Table 9). In the other groups, selected because of the higher standard deviations in spleen counts, a range of antibody levels occurred within each group and in no group was there a significant correlation between antibody levels and spleen counts. In a few animals (numbers 4 and 19), high levels of protection occurred despite levels of O antibodies no different from the mean level in the nonvaccinated controls (Tables 7 and 9).

DISCUSSION

B. abortus is a facultative intracellular parasite (11, 28), and evidence from the murine model of infection indicates that humoral (3, 26, 29, 32, 36, 39, 44) as well as cell-mediated (31, 37, 40) immune responses contribute to protection. The same views are currently held in respect to immunity against *Salmonella typhimurium* (16). In murine infections with both *B. abortus* (26, 32) and *S. typhimurium* (12), monoclonal antibodies specific for the OPS have been shown to confer protection passively. The data presented here complement and extend these findings with *B. abortus* by demonstrating that an acquired immune response to the OPS provides protection equivalent to that obtained with a living vaccine (Tables 7 and 9). It must be emphasized in this connection that in the model system we used, critical comparisons in logs of protection (i.e., the difference in mean log numbers of brucellae in the spleens of vaccinated and control groups) achieved by various vaccines must be restricted to comparisons made within the same experiment. For example, in experiments performed 15 months apart which are reported in Tables 3 and 7, vaccination of mice with 30 μg of the same preparation of porin-S-LPS in PBS produced logs of protection at 1 week p.i. of 3.33 and 1.74, respectively. Each result reflected a highly significant difference from control values ($P < 0.001$). Different lots of challenge strain 2308 were used in the two experiments. Small differences in lots of *B. abortus* challenge strains and in lots of experimental animals probably account for most of these variations. We have also noted that protection achieved at 1 week p.i. is a reliable predictor of protection at later time intervals. With infrequent exceptions, protection following vaccination with either living strain 19 or subcellular vaccines decreases between 1 and 4 weeks p.i. (Tables 2, 3, 4, and 6) (31), and long-term studies have demonstrated that even in mice vaccinated with strain 19, chronic infection develops following challenge infection with 5×10^4 CFU of strain 2308 and the protective effects of vaccination have disappeared by 12 weeks p.i. (A. J. Winter and G. E. Rowe, unpublished data).

The synthetic vaccine which contained long OPS chains proved better than the one with short OPS chains. This was associated in the long-chain vaccine with a higher apparent content of OPS (Table 1) and a more effective stimulation of O antibodies, particularly evident following a single vaccination (Table 7, day 28). The interpretation of data from the competitive inhibition assay must, however, be qualified in comparing preparations with long and short OPS chains. The short oligosaccharides may have combined less effectively with the monoclonal antibody used in the assay, which would have resulted in an underestimation of carbohydrate content. The quantity of O saccharide measured in the short-chain synthetic vaccine was nevertheless equal to that in the natural complex (Table 1), so that the differing qualities of the synthetic vaccines more likely reflected differences in OPS chain length. In a similar study performed with *S. typhimurium*, Svenson et al. (46) protected mice

TABLE 6. Vaccination with rough strain porin alone or complexed with long-chain OPS[a]

Vaccine (μg)	Log_{10} brucellae in spleens (mean ± SD)	
	1 wk p.i.	4 wk p.i.
None	6.17 ± 0.12	7.19 ± 0.43
R-porin[b] (30)	6.43 ± 0.47 (NS)	7.26 ± 0.14 (NS)
R-porin-OPS (long)[c] (30)	4.48 ± 0.25***	5.77 ± 0.90*

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; *, $P \leq 0.05$; NS, not significant. No adjuvant was used.

[b] Porin from rough strain RB51, denatured in sodium dodecyl sulfate.

[c] Long-chain OPS covalently linked to porin from strain RB51.

TABLE 7. Relationship between serum antibodies and protection against *B. abortus* in mice immunized with living or subcellular vaccines[a]

Vaccine (μg)	Antigen[b]	Antibody level (10^3) on expt day[c]:				Log_{10} brucellae in spleens (mean ± SD) (1 wk p.i.)
		0	28	35	42	
None	f6	2 (<)	2 (<)	3 (<)	36 (50)	6.39 ± 0.18
	Porin-R-LPS	4 (<)	3 (<)	6 (<)	6 (<)	
Strain 19[d]	f6	2 (<)	48 (100)	84 (200)	142 (400)	4.53 ± 0.84***
	Porin-R-LPS	3 (<)	3 (<)	5 (<)	6 (<)	
Porin-S-LPS[e] (30)	f6	2 (<)	98 (200)	233 (800)	229 (800)	4.64 ± 0.20***
	Porin-R-LPS	3 (<)	7 (<)	20 (25)	10 (<)	
Porin-R-LPS[e] (30)	f6	2 (<)	4 (<)	9 (<)	117 (200)	5.66 ± 0.87*
	Porin-R-LPS	4 (<)	5 (<)	9 (<)	13 (<)	
R-porin-OPS (long)[f] (30)	f6	2 (<)	137 (400)	190 (400)	235 (800)	4.54 ± 0.14***
	Porin-R-LPS	6 (<)	8 (<)	10 (<)	12 (<)	
R-porin-OPS (short)[f] (30)	f6	3 (<)	4 (<)	57 (100)	97 (200)	5.06 ± 0.78***
	Porin-R-LPS	4 (<)	5 (<)	9 (<)	10 (<)	

[a] Vaccination with subcellular products was performed s.c. 1 and 5 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. Strain 19 was inoculated 5 weeks before challenge. In comparisons with untreated control groups, ***, $P \leq 0.001$; *, $P \leq 0.05$.
[b] Antibodies were measured against either *B. abortus* S-LPS (f6) or rough strain RB51 native porin (porin-R-LPS).
[c] Mice were bled retro-orbitally prior to vaccination (day 0), revaccination (day 28), challenge (day 35), and spleen culture (day 42). Pooled serum samples were tested in kinetics-based ELISA. Numbers outside parentheses represent slope values. Numbers in parentheses are titers estimated from slopes (Fig. 1). <, titer < 25.
[d] Living *B. abortus* 19 at approximately 5×10^4 CFU per mouse.
[e] Undenatured porins from smooth strain (S) or rough strain RB51 (R) of 2308.
[f] Short- or long-chain OPS covalently linked to porin from strain RB51.

against lethal infection with synthetic vaccines containing octasaccharides of the OPS. However, no comparison was made in that study with vaccines containing longer OPS chains.

The weight of evidence supports the view that protection induced by the subcellular vaccines resulted from the induction of O antibodies. In the first instance, highly effective protection could be induced without the requirement for adjuvants which enhance cell-mediated immune responses (Tables 2, 3, 4, 6, and 7). Second, antibodies specific for moieties other than the OPS (e.g., porin, lipid A, or LPS core epitopes) were found in extremely low concentrations in sera of immune mice (Table 7). Third, there was a proportional relationship between the capacity of a vaccine to induce O antibodies and its capacity to induce protective immunity. This was evident in assays performed both before (Tables 4, 7, and 8) and after (Tables 7 and 8) challenge infection. Although a significant inverse correlation occurred within treatment groups between mean concentrations of O antibodies and mean numbers of *B. abortus* in spleens ($P < 0.05$) (Table 7), analysis of individuals within treatment groups failed to demonstrate such a relationship in all cases (Table 9). Examples are evident of both mice with a high level of protection in the absence of increased O antibodies (Table 9, mice 4 and 19) and others in which protection was marginal or absent despite raised levels of O antibodies (Table 9, mice 6, 10, 11, 12, and 24). Such exceptions do not, however, invalidate the hypothesis that O antibodies have a

TABLE 8. Comparison of antibody levels measured in ELISA with S-LPS (f6) and O glycolipid (O gly) antigens[a]

Vaccine (μg)	Antibody level (10^3) on expt day[b]:			
	35		42	
	f6	O gly	f6	O gly
Strain 19 (5×10^4 CFU)	84	70	142	93
Porin-S-LPS (30)	233	215	229	177
Porin-R-LPS (30)	9	8	117	80
R-porin-OPS (long) (30)	190	213	235	198
R-porin-OPS (short) (30)	57	50	97	77
None (PBS)	3	4	36	28

[a] Serum pools tested were from the same experiment reported in Table 7.
[b] Slope values from kinetics-based ELISA.

TABLE 9. Serum antibodies specific for OPS and numbers of *B. abortus* in spleens of individual mice 1 week after challenge infection[a]

Vaccine (μg)	Mouse no.	Brucellae in spleens (10^4)[b]	Antibody levels[c]	
			Slope (10^3)	Titer
Strain 19 (5×10^4 CFU)	1	0.6	50	100
	2	1.3	165	400
	3	1.4	103	200
	4	1.4	30	50
	5	8.3	32	50
	6	120.0	104	200
Porin-R-LPS (30)	7	5.6	55	100
	8	8.5	82	200
	9	10.0	75	200
	10	160.0	68	100
	11	180.0	131	200
	12	630.0	89	200
Porin-OPS (long) (30)	13	1.8	196	400
	14	3.3	178	400
	15	3.7	158	400
	16	4.0	196	400
	17	4.1	205	400
	18	4.7	209	400
Porin-OPS (short) (30)	19	2.7	18	25
	20	2.9	64	100
	21	5.5	112	200
	22	6.4	77	200
	23	25.0	25	25
	24	310.0	75	200

[a] Sera were those taken on day 42 in the experiment reported in Table 7.
[b] Mean number of *B. abortus* in spleens of the control group was 245×10^4.
[c] Slope values from kinetics-based ELISA with O glycolipid as the antigen. Titers were estimated from slopes (Fig. 1).

critical role in protective immunity. A more critical test of this hypothesis would be to determine the correlation between antibody titers just prior to challenge and protection. Moreover, the resolution of this question will require not only quantitative measurements but also comparisons of isotype distribution and functional assessments of O antibodies from mice exhibiting different levels of immunity.

There is growing evidence that porins or other outer membrane proteins will prove to be useful as vaccines capable of inducing protective antibodies against *Neisseria meningitidis* (7, 17), *Pseudomonas aeruginosa* (18, 30), and *Haemophilus influenzae* (19, 27). A protective role of porin antibodies against *S. typhimurium* has been proposed in several studies (4, 24, 49). Although some workers have now concluded that protection against *S. typhimurium* attributed to porin antibodies was due to O antibodies (41), the issue remains unresolved (49). Data reported here offer no evidence for the participation of porin antibodies in protection against *B. abortus*. The antibody response of mice to porin was almost negligible, even when porin vaccines lacked OPS (Table 7). The protection produced in mice following two vaccinations with porin from rough strain RB51 was almost certainly due to O antibodies. The data indicate that two inoculations of this vaccine primed the mice to produce an accelerated O antibody response to the challenge infection (Tables 7 and 9). It cannot be excluded that some of the antibodies reactive with f6 or O glycolipid were directed at core determinants. However, the marginal reactivity of these sera with porin-R-LPS (Table 7) indicates that the majority of antibodies were specific for OPS. Priming could have been a response to the porin and to trace quantities of OPS in the LPS of the rough strain or, in the absence of S-LPS, to the porin carrier alone. Whereas antibodies specific for porin and the group 3 outer membrane protein of *B. abortus* can be induced in cattle vaccinated with a rough strain of *B. abortus* such as 45/20 in TDM and MDP adjuvants (53, 55), experimental infection with smooth virulent strains fails to evoke an appreciable increase in outer membrane protein-specific antibodies in cattle (C. L. Baldwin, Ph.D. thesis, Cornell University, Ithaca, 1983) or mice (Table 7). This suggests that in the cell envelope of *B. abortus*, epitopes of OPS are strongly dominant over those of the associated outer membrane proteins. Moreover, the failure of monoclonal antibodies specific for the porin of *B. abortus* to protect mice (32) and of polyvalent antisera specific for porin or group 3 antibodies to agglutinate smooth whole *B. abortus* cells (A. J. Winter, unpublished data) suggest that, as in the members of the family *Enterobacteriaceae* (50), the outer membrane proteins of *B. abortus* are in their native state inaccessible to antibodies because of the long OPS chains. If this was so, antibodies protective against *B. abortus* would be limited to those with specificity for the OPS.

Although under natural conditions of infection the porin of *B. abortus* is ineffective in inducing antibodies, it does stimulate T-cell responses. Thus, peripheral blood lymphocytes of cattle infected with strain 2308 underwent blastogenic transformation when exposed to highly denatured porin proteins largely depleted of LPS (2). Denatured porin proteins also induced delayed-type hypersensitivity in cattle infected with strain 2308 or in mice infected with strain 19 (A. J. Winter, G. E. Rowe, and W. L. Castleman, unpublished data). The data from the present study do not, however, serve to advance the hypothesis that the porin of *B. abortus* can induce a protective cell-mediated immune response. Adjuvants were selected which enhanced cell-mediated as well as humoral immune responses (1, 9, 25, 33), and the combinations of TDM and MDP (53–55) and L-121 and MDP (A. J. Winter and G. E. Rowe, unpublished data) were already known to be potent in induction of cell-mediated immune responses against porins of *B. abortus* in cattle. These adjuvants did enhance the formation of O antibodies (Table 4), but evidence of protection attributable to cell-mediated immunity could not be inferred from any of these data. Although adoptive transfer experiments conducted in several laboratories have provided evidence for the existence of immune T cells which confer protection against *B. abortus* (31, 37, 40), the antigenic specificities of these T cells have not been established. A rigorous examination of the hypothesis that vaccination of mice with porins or other purified cell envelope proteins in conjunction with selected adjuvants can induce T cells capable of conferring protective immunity against *B. abortus* is currently under way in our laboratory.

The application of these findings from the mouse model to the construction of subcellular vaccines for cattle is still premature. Two recent well-controlled studies have failed to demonstrate protection against brucellosis in pregnant heifers by immunization with subcellular vaccines with or without S-LPS. Confer et al. (13) vaccinated cattle with a mixture of salt-extractable proteins (47) which did contain S-LPS as evidenced by the development of positive serological tests (13), whereas Adams et al. (L. G. Adams, R. P. Crawford, T. A. Ficht, R. Smith III, B. A. Sowa, J. W. Templeton, J. D. Williams, and A. M. Wu, 40th Annual Brucellosis Conference, Chicago, Ill., Nov. 14 and 15, 1987) used LPS-free recombinant outer membrane proteins of *B. abortus*. The failures of these vaccines may have had multiple causes and do not resolve the question of the importance of the OPS in an effective vaccine against bovine brucellosis.

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Squalene and Squalane Emulsions as Adjuvants

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Microfluidized squalene or squalane emulsions are efficient adjuvants, eliciting both humoral and cellular immune responses. Microfluidization stabilizes the emulsions and allows sterilization by terminal filtration. The emulsions are stable for years at ambient temperature and can be frozen. Antigens are added after emulsification so that conformational epitopes are not lost by denaturation and to facilitate manufacture. A Pluronic block copolymer can be added to the squalane or squalene emulsion. Soluble antigens administered in such emulsions generate cytotoxic T lymphocytes able to lyse target cells expressing the antigen in a genetically restricted fashion. Optionally a relatively nontoxic analog of muramyl dipeptide (MDP) or another immunomodulator can be added; however, the dose of MDP must be restricted to avoid systemic side effects in humans. Squalene or squalane emulsions without copolymers or MDP have very little toxicity and elicit potent antibody responses to several antigens in nonhuman primates. They could be used to improve a wide range of vaccines. Squalene or squalane emulsions have been administered in human cancer vaccines, with mild side effects and evidence of efficacy, in terms of both immune responses and antitumor activity. © 1999 Academic Press

The only adjuvants widely employed in human vaccines are aluminum hydroxide and aluminum phosphate. While these have been very useful in augmenting T helper cell type 2 (Th2) humoral immune responses to bacterial toxoids and other antigens, they have limitations. Aluminum compounds have not shown adjuvant effects when used with some antigens, such as influenza virus hemagglutinin (HA), *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxin conjugate, and typhoid vaccine (1). Furthermore, in humans as well as experimental animals, antigens adsorbed to aluminum salts more frequently elicit IgE antibody responses than in the absence of these salts; IgE antibodies may produce hypersensitivity to vaccine antigens (1). However, the principal limitation of aluminum salts is their inability to elicit cell-mediated immunity (CMI), including cytotoxic T-lymphocyte (CTL) responses (1, 2), which may be important in resistance against some microbial agents and tumors.

The traditional adjuvant eliciting CMI and CTL responses is Freund's complete adjuvant (FCA), a water-in-mineral oil emulsion containing mycobacteria. FCA produces granulomas at injection sites and is unacceptable for use in vaccines. Both the mineral oil and Arlacel A (mannide monooleate) components of Freund's adjuvants have produced tumors in mice under some conditions (3, 4).

The objective of the Syntex adjuvant program was to develop an alternative to FCA that would be suitable for use in a wide range of human and veterinary vaccines. The program was based on two premises: (a) a biocompatible oil should be used rather than mineral oil; and (b) the emulsion should be oil-in-water rather than water-in-oil, because the latter produces a long-lasting depot at the injection site. Several metabolizable oils (coconut, peanut, and olive oils, and lanolin) were screened as alternatives to mineral oil, and several detergents were tested as substitutes for the Arlacel A in Freund's adjuvants. Emulsions of squalene or squalane in an aqueous phase stabilized with polysorbate 80 were found to be efficacious adjuvants. Addition of Pluronic L121, and in some situations a relatively non-toxic analog of muramyl dipeptide, was found to augment adjuvant activity (5). Microfluidization was then shown to increase the stability of the squalane or squalene emulsions, leading to Syntex Adjuvant Formulation (SAF) (6). Later, alternative formulations of microfluidized squalene-in-water emulsions were developed by other investigators.

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DESCRIPTION OF METHODS

Squalene and Squalane Emulsions

Squalene (Fig. 1) is a linear hydrocarbon precursor of cholesterol found in many tissues, notably the livers of sharks (*Squalus*) and other fishes. Squalane is prepared by hydrogenation of squalene and is fully saturated (Fig. 1), which means that it is not subject to autoxidation. Small quantities of squalane are found in sebaceous secretions, so it is a natural product. Both squalene and squalane can be metabolized and have a good record in toxicology studies (7). Yarkoni and Rapp (8) had used squalene, as well as hexadecane and other lipids, with mycobacterial cell walls, to augment nonspecific immunity against tumors. As far as we are aware, ours were the first studies adding antigens to squalene and squalane emulsions and demonstrating their adjuvant activity (5). Squalene and squalane were found to be equally effective, and squalane was preferred because of its greater stability. Squalane is a free-flowing oil and has been used in pharmaceuticals and as a skin lubricant, as an ingredient in suppositories, and as a vehicle for lipophilic drugs. The squalane in SAF conforms with National Formulary guidelines and is used at a final concentration of 5% (w/v).

Polysorbate 80 (Tween 80, final concentration 0.4%) is the emulsifier in SAF. It is widely used as a surfactant in foods, cosmetics, and pharmaceuticals, including parenteral formulations. Surfactants are classified according to their relative affinity for water and lipid, or hydrophile–lipophile balance (HLB). The HLB of Polysorbate 80 is 12–16, so it is very stable in aqueous media. The Polysorbate 80 used in SAF conforms with U.S. Pharmacopoeia guidelines. This surfactant maintains the stability of microfluidized squalene or squalane emulsions for years. The aqueous continuous phase of SAF comprises isotonic phosphate-buffered saline (PBS) with a final formulation pH 7.4.

Microfluidization

Squalene and squalane emulsions were initially prepared by blending components in a laboratory vortex.

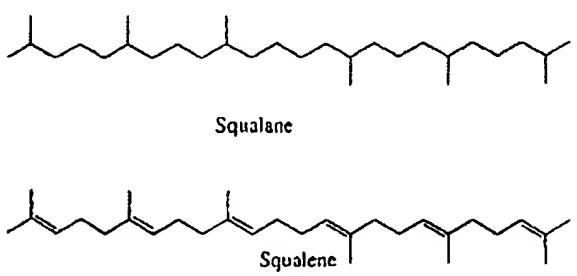

FIG. 1. Structures of squalene and squalane.

This method was unsuitable for large-scale production. After evaluation of the various equipment options available for the manufacture of emulsions the Microfluidizer was selected (6). With this instrument the emulsion is formed as two fluidized streams interact at high velocities in an interaction chamber. The Microfluidizer is driven by air or nitrogen and can operate at internal pressures of 20,000 psi with a throughput of 300–500 μl/min. The emulsion can be continuously recycled through a closed-loop system. Particle size analysis showed that with seven cycles through the Microfluidizer the mean droplet size was 165 nm with a range of 81–270 nm. When the number of larger particles is small, it becomes possible to filter the emulsion through a 0.22-μm filter membrane, thus allowing terminal filtration (6). Microfluidization has three advantages: (1) the stability of the emulsion is increased; (2) it allows terminal filtration, the preferred way to ensure sterility of the final product; and (3) submicrometer oil particles can pass from injection sites into the lymphatics and then to lymph nodes of the drainage chain, blood and spleen. This reduces the likelihood of establishing an oily depot at the injection site, which may produce local inflammation.

The stability of microfluidized SAF is remarkable. After 6 years at ambient room temperature, the emulsion was found to remain physically stable (6). The emulsion could be frozen and kept at −20°C without phase separation on thawing. After long storage emulsions tend to "cream," with lighter oil particles rising above the denser continuous phase, but this is not due to coalescence of the particles and physical mixing restores the milky appearance and adjuvant activity.

Is a Depot of Antigen at the Injection Site Required?

Glenny *et al.* (9), who introduced aluminum adjuvants, postulated that they act by forming depots at injection sites, thereby prolonging the duration of interaction between antigens and responding cells. In the regional lymph nodes of rabbits immunized with soluble diphtheria toxoid, antibody-forming lymphocytes were found to disappear after 3 weeks, whereas when the antigen was adsorbed to aluminum phosphate antibody-forming cells persisted longer than 4 weeks (10). However, Holt (11) showed that antibody formation continued even after removal of the adjuvant–antigen depot from the site of injection. Similarly, Freund (12) found that excision of the injection site of antigen with his adjuvant 8 to 10 days after injection had little effect on the magnitude or duration of the immune response.

These observations suggest that the association of antigen with antigen presenting cells in responding lymphoid tissues is more important than having a depot at the injection site. Dendritic cells can carry antigen for presentation to T lymphocytes, and antigens, or immune complexes, can be retained in association with

the projections of follicular dendritic cells for months; these cells efficiently present antigens to B lymphocytes (13).

One of the reasons for using small oil-in-water particles carrying antigens is that they are efficiently endocytosed by antigen presenting cells. A second reason is that small particles can readily pass from injection sites into lymphatics. The cutoff is quite sharp, as demonstrated with liposomes containing radioactive markers. Five hours after injection of small liposomes (<500 nm, neutral or negatively charged) they were found in lymphatics and, after 30 hours, they were concentrated in lymph nodes (14). Larger liposomes did not pass into lymph and remained localized at injection sites much longer.

Nonionic Block Copolymers

These surface-active polymers comprise hydrophilic blocks of polyoxyethylene (POE) and hydrophobic blocks of polyoxypropylene (POP) in various configurations, proportions, and relative molecular masses (Fig. 2). Hunter and colleagues (15) showed that some triblock copolymers have adjuvant activity when added to mineral oil. Allison and Byars (5) found that Pluronic L121, added to squalane emulsions in a final concentration of 5% (w/v), augmented their adjuvant effect. Pluronic L121 (also termed poloxamer 401) contains 10% by weight of POE and has an average M_r 4400. It is liquid at room temperature and has a lipophile–hydrophile balance close to 1.0. Thus L121 is poorly soluble in water, does not stabilize emulsions, and is termed a spreading agent. It adheres to the surface of oil droplets in aqueous media, where it can increase the concentration of proteins at the interface (16). Some antigens have been demonstrated at this site by immunogold electron microscopy (5). Through binding activating components rather than inhibitors, oil droplets with L121 activate complement (16). It has long been known that activated complement facilitates the localization of antigens on follicular dendritic cells (FDCs) and the induction of B memory (13). A fragment of C3, designated C3d, binds CR2 (CD21) on FDCs and B lymphocytes (17); CD21 associates with CD19 in B cells and amplifies their proliferative responses. Thus targeting of antigens to FDCs, through binding of C3d to CD21, may be an important requirement for eliciting antigen-specific B cell proliferation and thereby establishing immunological memory.

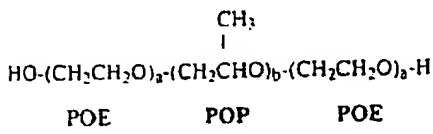

FIG. 2. Generalized structure of a Pluronic triblock copolymer, showing blocks of polyoxyethylene (POE) and polyoxypropylene (POP).

Muramyl Dipeptide Analogs

The adjuvant-active component of mycobacterial cell walls was shown by Ellouz *et al.* (18) to be muramyl dipeptide (MDP). When added to Freund's incomplete adjuvant, MDP allowed it to function like the complete adjuvant, eliciting delayed hypersensitivity in the guinea pig. MDP is pyrogenic, and produces uveitis in rabbits and arthritis in rats. Several laboratories synthesized analogs of MDP to obtain compounds with comparable adjuvant activity but reduced pyrogenicity and other side effects. The analog selected by Syntex was *N*-acetylmuramyl-L-threonyl-D-isoglutamine (Thr-MDP) (5) (Fig. 3). Other analogs include GMDP [*N*-acetylglucosaminyl-(1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine] (19), and MTP-PE, with phosphatidylethanolamine linked to MTP (20). Thr-MDP has been added to SAF to augment CMI and CTL responses; the final dose in humans should not exceed 0.4 mg.

Muramyl peptide analogs have other activities. For example, MTP-PE increases nonspecific resistance to infectious agents and tumors, which Thr-MDP does not. GMDP stimulates the recovery of the bone marrow following administration of cytotoxic drugs or radiation. Whether these additional activities can be achieved without limiting side effects in humans remains to be established.

Addition of Antigens to Preformed Emulsions

The standard practice reached early in the Syntex adjuvant program has been to add antigens to preformed squalene or squalane emulsions. This is done for two reasons. First, it is convenient to prepare a large batch of stable emulsion under good manufacturing processing (GMP) conditions and add antigen later. If the antigen is unstable, it can be reconstituted just before injection into recipients. Second, when protein antigens are present during emulsification, they can be

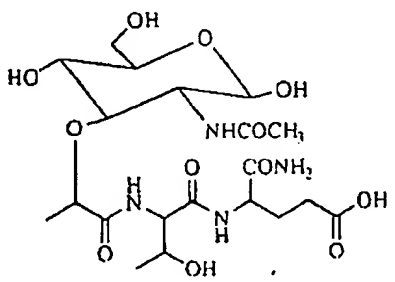

FIG. 3. Structure of *N*-acetylmuramyl-L-threonyl-D-isoglutamine, a relatively nontoxic muramyl dipeptide.

denatured by shearing forces. We found that when bovine serum albumin was emulsified in the presence of oil and injected into experimental animals, a large proportion of antibodies elicited recognized determinants within the protein (21). When the same antigen was mixed with preformed squalane emulsions, the antibodies elicited bound almost exclusively to determinants on the surface of the native protein. The implication is that when antigens are present during emulsification conformational epitopes can be lost, and many of the epitopes involved in protective immunity are conformational.

Isotypes of Antibodies Elicited

Antibodies of any isotype, having a reasonably high affinity for antigens and present in sufficient quantity, can neutralize bacterial toxins and even some viruses (22). However, antibodies of some isotypes efficiently activate complement, bind to high-affinity receptors on monocytes, and act synergistically with antibody-dependent effector cells (ADCCs) to produce cytotoxicity. Examples are IgG2a antibodies in mice and IgG1 antibodies in humans, both of which bind to high-affinity FcγI receptors. Studies with isotype-switch variants of murine monoclonal antibodies (which have the same Fab regions, so binding to antigen is comparable) show that IgG2a antibodies confer better protection against tumors than those of other isotypes (23). Studies with "reshaped" human antibodies, genetically constructed to have antigen-binding hypervariable regions like those of rodent monoclonals, confirm the superiority of the human IgG1 isotype in ADCC-mediated lysis (24). The desirability of developing an adjuvant formulation that preferentially elicits high-affinity antibodies of the IgG2a isotype in mice and IgG1 in humans is apparent.

Antigens with aluminum salts elicit Th2-type responses, with high concentrations of IgG1 in the mouse (1, 21). When SAF was used, most of the antibody was found to be of the IgG2a isotype (21). SAF elicits Th1-type responses, with relatively high production of IFNγ, which preferentially stimulates the formation of IgG2a antibodies.

Cell-Mediated Immune Responses

When antigens were administered to guinea pigs in SAF, strong delayed-type hypersensitivity was elicited (5). In guinea pigs immunization with recombinant herpes simplex GD-2t in SAF generated cytotoxic T cells that lysed target cells expressing viral antigens in a genetically restricted fashion (25). The soluble antigen ovalbumin in SAF elicited cytotoxic T cells, which led to regression of ovalbumin-transfected EG7 tumor cells (26).

Examples of the Efficacy of SAF

Influenza virus hemagglutinin in SAF was found to elicit higher antibody responses than were obtained by the currently used subunit preparation (27). Vaccination in persons aged 65 years or older is recommended because influenza is a severe disease in the elderly, but about one-third of aging recipients do not demonstrate antibodies following administration of HA. Responses in aging mice are also inconsistent, but SAF makes them much more uniform (27). SAF also augmented antibody formation by hepatitis B vaccine, even in low-responder strains of mice; it was possible to reduce the dose of antigen by a factor of 10 and to obtain consistent responses with two, instead of the conventional three, doses (28). This could decrease the cost of an important vaccine, especially in developing countries.

A guinea pig model of genital herpes simplex virus (HSV) infection was studied. Immunization with recombinant GD-2t in SAF provided strong protection against viral challenge, including reduction of local lesion size and the establishment of dorsal root ganglion infection, a source of recurrence (25). Epstein–Barr virus (EBV) causes a lymphoproliferative disease in cotton-top tamarins; the gp340 viral surface antigen in SAF was found to protect recipients of EBV, whereas alum-adjuvanted viral antigen did not (29).

SAF with a formalin-inactivated whole-virus vaccine protected macaque monkeys against challenge with simian immunodeficiency virus (30). The gp120 antigen of human immunodeficiency virus in SAF protected chimpanzees against challenge with the virus (31).

Periodontitis is a common disorder in which the attachment tissues of the teeth and their alveolar bone housing are destroyed, resulting in tooth loss. The gram-negative anaerobic bacterium *Porphyromonas gingivalis* has been associated with severe forms of periodontitis. Immunization of the primate *Macaca fascicularis* with killed *P. gingivalis* in SAF was found to inhibit progression of periodontal tissue destruction (32). These findings show that intramuscular and subcutaneous vaccination can protect a mucosal tissue. A vaccine against periodontal disease could have a major impact in dentistry.

Two murine models of malignant disease were studied: idiotypic vaccination against B lymphomas (33) and an anti-idiotypic monoclonal antibody mimic of an antigen expressed on melanoma cells (34). Antigens in SAF were shown to elicit humoral and cellular responses to the antigens used and resistance to tumor cell challenge.

Cancer Patients

The success of the experimental studies on mouse B lymphomas provided justification for using SAF in hu-

man tumor vaccine trials. These have demonstrated the activity of the adjuvant formulation in humans, and provided useful information about side effects, that cannot be predicted from findings in experimental animals. Chemotherapy and radiation therapy can induce remissions in patients with B-cell lymphomas, but most patients relapse and cannot be cured by these therapies. The idiotypic surface immunoglobulin of these tumor cells can be used as a tumor antigen, which is coupled to a carrier, such as keyhole limpet hemocyanin. In the absence of adjuvant, anti-idiotypic responses are poor. In the presence of SAF, about one half (49%) of immunized patients had specific immune responses against the idiotypes of their tumor immunoglobulin (35). Patients in remission mounting an immune response showed significantly improved freedom from progression compared with those not demonstrating an immune response (7.9 years as compared with 1.3 years, $P < 0.0001$). Some patients received SAF with and others without Thr-MDP (the threonyl analog of MDP). Reactions at injection sites (erythema, tenderness, induration), as well as myalgia, arthralgia, and fever, were related to the dose of Thr-MDP. The maximum tolerated dose was found to be 0.4 mg. The side effects were transient, lasting 24–48 h.

Active specific immunotherapy of metastatic melanoma with an anti-idiotype vaccine in SAF was attempted by Quan *et al.* (36). Twenty six patients were studied, 17 of whom had previously received chemotherapy. Computed tomographic scans of the chest, abdomen, and pelvis and magnetic resonance imaging of the brain were used to evaluate treatment responses. Fever, myalgia, arthralgia, fatigue, nausea, and headaches were observed in patients with higher doses of Thr-MDP; doses of 100 μg were well tolerated. This dose was sufficient to elicit antibodies against the idiotypic vaccine, a response associated with a statistically significant clinical antitumor effect in six patients. One patient developing a specific antibody against the melanoma epitope, (Ab)3, had a complete clinical response and had survived for 5 years when the paper was written.

These observations suggest that the side effects of SAF without Thr-MDP are mild and that it may be a useful adjuvant for cancer vaccines. Doses of Thr-MDP up to 100 μg are tolerable in cancer patients. The major mechanism(s) effecting immunity against tumors is (are) unknown and may vary among tumors. *In vitro* antibodies can certainly exert antitumor effects, either lysing tumors (24) or inhibiting their replication (37). However, soluble antigens in SAF, with or without Thr-MDP, can also elicit CTL responses, lysing target cells in a genetically restricted fashion and exerting antitumor immunity in experimental animals (25, 26).

Other Squalene and Squalane Emulsions

MF-59

This adjuvant formulation was developed by Ott and collaborators of the Chiron Corporation (20). It was originally used as a way to administer the muramyl tripeptide MTP-PE, which is amphiphilic. The composition is 5% (w/v) squalane, 0.5% sorbitan monooleate and 0.5% sorbitan trioleate. The squalene-in-water emulsion is prepared by microfluidization. MF-59 was shown to increase antibody responses to a variety of antigens, including GD-2t of herpes simplex virus, HIV, and influenza virus hemagglutinin (20). MF-59 has been evaluated in humans in an HIV vaccine (38) and an influenza vaccine (39). Significant toxicity was attributed to MTP-PE. MF-59 cannot be frozen and may not be as stable as other squalene or squalane formulations (40).

Antigen Formulation

Also known as AF or SPT, this formulation was developed by the IDEC Pharmaceuticals Corporation (41). It differs from SAF in that the concentration of Pluronic L121 is reduced (1.25%); muramyl dipeptide is not used. When soluble proteins were administered to mice in AF, antigen-specific cytotoxic T cells were induced (41).

Hjorth Adjuvant Formulation 1

Richard Hjorth of Wyeth Laboratories has devised a stable microfludized emulsion of the following composition: squalene 10%, lecithin 1%, and Tween 80 0.2% (42). It was designed to use only ingredients that have been routinely and safely injected into humans and to have minimal local and systemic effects. Lecithin is the emulsifier of a soybean oil preparation (Intralipid) that has been administered to many infants. The lecithin in the adjuvant formulation is the same as a vehicle for injectable Bicillin, which has been used in humans for years. Hjorth Adjuvant Formulation 1 is microfluidized and sterilized by terminal filtration. This adjuvant formulation is effective in eliciting high antibody titers with a wide range of antigens in experimental animals, including monkeys. For example, administered with influenza virus hemagglutinin, the adjuvant formulation was found to generate antibody titers comparable to those obtained with other potent adjuvants such as SAF, and higher than those observed with liposomes, novasomes, and QS21. Reactions at injection sites in monkeys were slight and creatine phosphokinase measurements were within normal limits; there was no pyrogenicity and blood chemistry revealed no abnormality of liver or kidney function.

Ovalbumin administered to mice in Hjorth Adjuvant Formulation 1 elicited high levels of antibodies, principally of the IgG1 isotype, and no demonstrable CTL response, suggesting a Th2-type response (O. Wijburg,

unpublished). Whether this would also be true in humans remains to be determined.

Hjorth Adjuvant Formulation 2

Hjorth's second adjuvant formulation (43) has the following composition: squalene (5%), glycerol 20%, and Tween 80 (0.2%). It is prepared by microfluidization and can be stored or refrigerated for long periods with no effect on potency. This formulation cannot be sterilized by terminal filtration through a 0.2-μm filter. Hjorth Adjuvant Formulation 2, used with influenza virus hemagglutinin and other antigens in monkeys, has elicited high antibody responses. The relative efficiency of Hjorth's two adjuvant formulations, used with different antigens in human and veterinary vaccines, remains to be determined.

CONCLUDING REMARKS

The observations presented in this article suggest that SAF, without a muramyl peptide analog or with a low dose of the analog, may be efficacious in vaccines for human cancers and possibly serious infections such as HIV. Hjorth's squalene emulsions appear to be the least toxic formulations and may be useful with a wide range of antigens where antibody titers, irrespective of their isotype, play a major role in protection and CTL responses do not. For example, such an adjuvant could improve influenza vaccines, allowing the use of lower doses of antigen and augmenting responses in the elderly. It might allow reduction of the amount of hepatitis B virus surface antigen in vaccines and elicit protective responses with two instead of the currently used three injections. The adjuvant might augment responses to bacterial glycoconjugates as well as to other viral antigens.

In general, squalene and squalane emulsions offer good opportunities for those that have the courage to take them from laboratory investigation and develop routinely administered human and veterinary vaccines.

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APPENDIX C
RELATED PROCEEDINGS

None.